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CONTENTS

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No. 2, February, 1927

No. 3, March, 1927

No. 4, April, 1927

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PHILADELPHIA, PENNSYLVANIA, DECEMBER 28 TO 30, 1926

Headquarters, Bellevue Stratford Hotel

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GENERAL BACTERIOLOGY

1. *Study of the Growth of Cl. botulinum and Cl. sporogenes in Veal Infusion Broth under Reduced Pressures.* GAIL M. DACK, W. A. STARIN AND MARIE WERNER, Department of Hygiene and Bacteriology, University of Chicago.

Cl. botulinum types A and B and *Cl. sporogenes* were inoculated into tubes of veal infusion broth which were placed under reduced pressures of air, oxygen and carbon dioxide. The tubes were sealed under the

reduced pressures and incubated at 37°C. The growth of the organisms and the toxin production of *Cl. botulinum* were studied under these conditions. Both spores and vegetative cells were used.

At air pressures of 4 cm. and less growth was regular and occurred in most cases within one to two days. At pressures greater than 4 cm. growth was irregular and occurred in some cases after several months. No growth was obtained in any tube at pressures greater than 16 cm.

No marked difference was noted between the growth of spores and vegetative cells under reduced air pressures. Toxin was not demonstrated in greater dilutions where 0.8 cm. of pressure was employed than where 5 cm. was used.

Hydrogen peroxide was not demonstrated by Avery's method in any of the tubes tested.

At oxygen pressures of 5 and 10 cm. no growth was noted after three months observation.

Growth of the organisms occurred regularly and uniformly at carbon dioxide pressures as high as 50 cm. Pressures higher than this were not employed. Growth was no greater in tubes which were first evacuated and then placed under atmospheric pressure of carbon dioxide before finally reducing the pressures than in tubes which were merely evacuated and the final carbon dioxide pressure maintained.

Cultures tested after one, five- and thirty-day periods of growth showed toxin production to be at its maximum after five days. The highest count of organisms was obtained after the one-day period of growth.

The hydrogen-ion concentration of the cultures remained within the limits or growth for each organism tested throughout the entire experiment.

2. *The Isolation of Pathogenic Anaerobes from African Poisoned Arrows.*

Ivan C. Hall, University of Colorado, Denver, Colorado.

The literature on poisoned arrows indicates that they owe their fatal action not only to various specific poisons but also in part to pathogenic microörganisms. It is well known that arrow wounds which are not fatal within a few minutes or hours are likely to become the seat of serious infections, notably tetanus, gaseous gangrene and cellulitis. Yet bacteriologic studies of such wounds have been few and no studies of the bacterial flora of the arrows themselves appear in the available literature.

My study deals with six arrows made by African bushmen and brought

back to America by the Denver African Expedition of 1925, which was headed by Dr. C. E. Cadle. Three of these arrows belonged to the Kalahari bushmen, two to the Heikum tribe, and one to the Ovachimbas of the Kaokoveldt. Only the Heikum arrows yielded poison; this was a dark, dry, gummy substance and, according to Dr. Richard Whitehead belongs to the group of glucosides, of which ouabain is an example, found in certain species of *Strophanthus*. A small guinea pig was killed in six minutes by 10 mgm. of the crude poison.

All of the arrows except the smaller Heikum arrow had pathogenic bacteria upon their points. Aside from several species of "hay bacilli," I have isolated and identified the non-pathogenic *B. centro-sporogenes*, *B. bifermentans*, *B. sporogenes*, two species of sporulating anaerobes belonging to obscure or possibly new species, *Staphylococcus albus*, *Streptococcus fecalis* and *Streptococcus mitis*. The pathogens were *B. histolyticus*, *B. Novyi*, *B. septicus*, and *B. Welchii;* all of these were typically virulent for guinea pigs. Special tests for *B. tetani*, *B. botulinus* and *B. coli* failed to reveal any of these germs.

3. Quantitative Aspects of the Protein Metabolism of C. putrefaciens (McBryde). L. B. PARSONS AND W. S. STURGES, Laboratory of the Cudahy Packing Company, Omaha, Nebraska.

A complete study has been made of the organism first isolated by McBryde (U. S. Dept. Agri., B. A. I. Bull. 132, 1911). The formol and ammonia nitrogen produced by *C. putrefaciens* in meat and gelatin media has been determined as a function of time. These values show this organism to be markedly proteolytic although other common putrefactive anaerobes are shown to digest more rapidly and produce greater amounts of ammonia and amino acids. Volatile acids produced during proteolysis consist of 85 to 90 per cent acetic and 10 to 15 per cent isovaleric. Non-volatile acids are not produced. An exact equivalence between volatile acid and ammonia has been demonstrated for six strains at all ages and for 24 strains at one age. Preliminary data indicate that this equivalence does not exist in the case of several other anaerobes. These data admit of certain conclusions of a fundamental nature concerning the mechanism of deamidization.

4. A Study of Some Anaerobic Microörganisms Found in Naso-pharyngeal Washings. SARA E. BRANHAM, Department of Hygiene and Bacteriology, University of Chicago.

Naso-pharyngeal washings from 26 persons were examined by the

technique of Olitsky and Gates. These 26 washings represented 8 normal persons, 13 with colds, and 5 cases of acute uncomplicated influenza within 36 hours after the onset. From 11 of these samples 17 strains of anaerobic microörganisms were isolated, 14 of which seemed to belong to species previously described. Five strains seemed to be identical with the *Staph. parvulus* reported first by Veillon and Zuber, and since found abundantly by Holman and Krock and by Hall and Howitt. Two were probably the spirochaete described by Tunnicliff. Seven were apparently the filterable microörganisms reported by Olitsky and Gates: 2 of these seemed identical with their Group I, 1 with Group III. Four were probably either *Bacterium pneumosintes* or Group II; 3 of these came from cases of influenza.

The three remaining strains seem to be hitherto undescribed. One of these, a small hemolytic diplococcus, growing only under anaerobic conditions, is described here.

5. *On the Cultural Characteristics of Lactobacillus acidophilus.* C. Roos, H. K. Mulford Company, Biological Laboratories, Glenolden, Pa.

The name *L. acidophilus* applies to a group of biologically related strains variable in cultural and morphological characteristics.

The changes noted in a strain under close observation for four years strongly indicate that the various types of *L. acidophilus* colonies, as found upon plating feces, are mutants.

Acid production, carbohydrate fermentation, colony formation are not constant characteristics and alone do not constitute a basis for classification.

From the standpoint of clinical usage, source of the strain, nutrient and physical requirements to facilitate growth, and temperature range are important factors and must necessarily be considered.

6. *Potato Utilization as Indicated by Hydrogen Peroxide. (Demonstration.)* Jean Güll and Jean Broadhurst, Teachers College, Columbia University, New York City.

The dissatisfaction commonly felt with solid potato as a diagnostic medium is due not only to the irregularity with which growth occurs on potato for a given species, but to the variations in appearance due to the differences in the softening of the potato in sterilizing, the height of the water in the tube and the "browning" and drying of the potato slices. We have adapted, therefore, a hydrogen peroxide test being used in our laboratory to this situation, adding 1 cc. of hydrogen perox-

ide to twenty-four hour (loop) cultures of the organism in (5 cc.) semi-fluid potato, stirring the mixture thoroughly after releasing the hydrogen peroxide at the bottom of the tube. The results with some thirty species of bacteria may be classified in several qualitative groups: (1) no ebullition of gas, as in sterile media, (2) a mere ring of surface bubbles, (3) a frothy layer of about $\frac{1}{6}$ to $\frac{1}{4}$ the height of the potato-hydrogen-peroxide mixture, (4) a foamy mass about equalling the height of the mixture, and (5) a foamy mass two to almost four times the height of the mixture, as in *Serratia marcescens* and *Pseudomonas pyocyanea*.

These qualitative groups are merely tentative; and this report is but a preliminary one, made in the hope of securing co-operation (especially cultures) for other phases of hydrogen-peroxide reaction (e.g., effect upon pigment manifestations, bacterial relationships) now being carried on in our laboratory.

7. *Apparatus Suggestions: (Demonstration), (1) Test Tube Plugs, (2) Semi-porous Petri Dishes, and (3) Centimeter Counters.* JEAN BROADHURST, Teachers College, Columbia University, New York City.

1. Test tube plugs. To decrease the labor and expense attendant upon the usual rolled cotton plug, a hollow rubber plug has been devised. The hollow is loosely filled with a small mass of absorbent cotton, insuring sterility but readily meeting the usual air and steam adjustments; each end of the plug curves slightly towards its central opening, causing the slightly bulging sides to press tightly against the test tube. A surfacing of paraffin, if desired, is easily applied, and it interferes less with the future manipulations of the plug than in the case of cotton plugs. If preferred a covering of absorbent cotton can be used on these rubber plugs; its application requires but a single manipulation in which the cotton center is also placed in the plug cavity.

2. Semi-porous Petri dishes. In an effort to secure a cheap semi-porous Petri dish cover several different chemical substances, such as cellulose acetate, and commercially known "fillers" and lacquers have been tried on the unglazed white covers. The method of application of the most satisfactory substances will be demonstrated, with exhibits of plates similarly treated at a cost of but a fraction of a cent each, and used continuously from one to four months.

3. The delimitation of given areas on slides for direct counting of bacteria (vaccines, milk) and on Petri dishes for colony counts may be more satisfactorily done by applying pieces of gummed paper, with

cut-out openings of one and two square centimeters. Such cut-out pasters are preferable to wax pencil outlines, because they are exact and there are no fragments of pencil to confuse the microscopic count. In counting crowded Petri dishes, several advantages are found: the cut-out paster fits close to the glass surface and changes in the angle of the worker's head cause less variation in the size of the field actually counted; the Petri dish can be held up in front of the eye, the worker in any comfortable position which can be varied at will, instead of bending in a cramped position over the Stewart or Wolffhuegel plate. The margin of the paster is wide enough to be used for recording results, and it acts as an eye shield, especially in the lantern-counting of colonies previously described by the author. The two-square centimeter counters are recommended for direct milk counts where a thinner film is desired than is obtained with the one-square centimeter area. Counters for trial use will be supplied on request.

8. *A Preliminary Study of Two Hundred Gram Negative Bacilli Isolated from Cases of Genito-Urinary Infection.* J. H. HILL, L. R. SEIDMAN, A. M. S. STADNICHENKO AND M. G. ELLIS, James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Md.

This paper is a study of the cultural characteristics of 200 cultures of Gram-negative bacilli isolated from genito-urinary infections. The methods used included the fermentation of carbohydrates, growth in gelatin, the production of acetyl-methyl-carbinol, the methyl red test, indole production, motility, urea decomposition, haemolysis, capsule formation and citrate utilization in Koser's citrate broths and on Simmons citrate agar.

From these observations the organisms were divided into five main groups as follows:

Group I. Organisms not producing acetyl-methyl carbinol, methyl red positive, fermenting lactose and other carbohydrates with the production of acid and gas, citrate utilization none or scant.

Group II. Organisms producing acetyl-methyl carbinol, methyl red negative, fermenting lactose and other carbohydrates with the production of acid and gas, citrate utilization prompt.

Group III. *Proteus*.

Group IV. *Pseudomonas pyocyanea*.

Group V. Organisms not in Groups I, II, III, or IV.

Under Group II a study of the rate of production of acetyl-methyl-carbinol has been made, under Group III the decomposition of urea has been studied especially, and the fermentative action of Group IV has been emphasized.

These organisms, especially Groups I and II have been compared with each other and with organisms previously reported by others in genito-urinary infections. Comparisons have also been made with similar organisms isolated by others from different sources, such as the intestinal tract, soil, milk, etc. Emphasis has been placed on the pathogenicity of the Group II organisms in our series. A correlation of the organisms studied with the clinical findings is included.

9. Surface Tension Studies with L. acidophilus and L. bulgaricus. Nicholas Kopeloff and Philip Beerman, Department of Bacteriology, Psychiatric Institute, Ward's Island, N. Y.

A standardization method has been developed yielding fairly concordant results in growing these lactobacilli at varying surface tensions. Using sodium ricinoleate and sodium oleate as depressants 9 different strains of *L. acidophilus* (6 of these isolated from commercial Acidophilus products now on the market) and 6 different strains of *L. bulgaricus* have been repeatedly tested in different concentrations.

All strains of so-called *L. acidophilus* with one exception were able to grow at a surface tension of three or more dynes lower than any *L. bulgaricus* in the presence of sodium ricinoleate. The average critical point for *L. acidophilus* strains for this depressant was 37.9 dynes, while the average critical point for *L. bulgaricus* strains was 5.2 dynes higher, or 43.1 dynes.

It is significant that two strains of *L. acidophilus* which have been passed through the human intestinal tract and are of proven therapeutic benefit had the lowest critical point, namely, 35 dynes.

Both *L. acidophilus* and *L. bulgaricus* strains grew at lower surface tension when sodium oleate was used as a depressant than when equivalent concentrations of sodium ricinoleate were employed.

These results substantiate the contention that surface tension is a satisfactory criterion for differentiating *L. acidophilus* from *L. bulgaricus.*

10. The Effect of Sodium Ricinoleate as a Surface Tension Depressant upon the Growth of Bacterium coli. W. R. Albus, Bureau of Dairy Industry, U. S. Dept. of Agriculture, Washington, D. C.

A study has been made of the effect of sodium ricinoleate as a surface

tension depressant and as a salt, upon the growth of a strain of *Bacterium coli.* The results of these experiments are presented.

11. The Effect of Bacterial Cells upon the Chemical Composition of the Surrounding Menstruum as Influenced by the Presence of Various Electrolytes. H. J. Shaughnessy and C.-E. A. Winslow, Yale School of Medicine, New Haven, Conn.

Cells of *Bact. coli* in the zone of physiological interest (pH 4.0 to 10.0) produce changes in acidity by means of direct absorption of H or OH ions in such a manner that there may be no injury to the cell. Sodium chloride and calcium chloride decrease the ability of the cells to perform this action,— most markedly in the alkaline range.

In addition to this type of regulation, there is another which seems to be dependent upon the production of carbon dioxide and ammonia, aided perhaps by diffusion of hydrogen ions carried into the surrounding fluid by chlorine or phosphate ions.

More prolonged exposure of bacterial cells to aqueous menstrua leads to an over-production of ammonia and in the case of *B. cereus* which dies under the conditions of the experiment, a marked excess of alkali is produced.

Living cells of *Bact. coli* and even dead cells of *B. cereus* appear to be relatively impermeable to chlorine phosphate and calcium ions. On the other hand carbon dioxide and ammonia pass freely. The cell wall of *B. cereus* is in general much more permeable than that of *Bact. coli.*

Neither heating the cells at 60° for fifteen minutes nor boiling for 30 minutes affects either the diffusion of electrolytes or the liberation of carbon dioxide. Boiling however almost wholly inhibits the production of ammonia.

Dilute solutions of sodium chloride promote the liberation of the substances studied from both normal and heated cells.

Strong solutions of sodium and of calcium on the other hand decrease the liberation of ammonia and other alkaline substances, but increase, relatively, the liberation of acidic substances.

In the case of *Bact. coli* very strong solutions of calcium show a very sharp initial rise in titratable alkalinity and in titratable acidity followed by a fall, which phenomenon we interpret as due to a decreased permeability leading to lysis and liberation of proteins followed by an accumulation of new reactive films on the protein micellae or to absorption of the oppositely charged ions.

12. *The Interpretation of Changes in Electrical Resistance Accompanying the Death of Cells.* A. ZOOND, MacDonald College, Quebec, Canada.

The electrical resistance of suspensions of *Bacillus cereus* in balanced Ringer solution is found to decrease when the suspension is killed by heat or by mercuric chloride. The resistance measurements, however, indicate that the phenomena accompanying the death of the cell by heat and by mercuric chloride are not identical.

When cells killed by mercuric chloride are resuspended by repeated centrifuging in Ringer solution, it is found that the resistance of the suspension is the same before and after death. This clearly indicates that the conductivity of bacterial cells, i.e. their resistance to the passage of ions moving under the influence of an electric current, is not materially affected by the death of the cell. The pronounced drop in resistance accompanying the death of the cells under the influence of mercuric chloride can only be accounted for by the assumption that death is accompanied by the destruction of the semipermeable membrane surrounding the bacterial cell, and that consequently there is an adjustment of osmotic differences which may modify the concentration of salts in the suspending solution, and thus affect its electrical resistance.

When a suspension of *Bacillus cereus* in Ringer solution is killed by heat, there is a very pronounced drop in the resistance of the suspension. This, however, is accompanied by a corresponding drop in the resistance of the suspending solution, indicating that the resistance of the cells is not materially affected by the heat treatment, but that the decrease in resistance is due to the diffusion of salts of the cell. This view is supported by the fact that when the suspending solution is replaced by a solution of normal concentration by means of repeated centrifuging the resistance of the suspension again rises to a value of little below the resistance of the living suspension. That it does not quite attain that value is explained by the observed fact that the volume of the cells is materially decreased by the heat treatment. This is further supported by the observation that when a suspension killed by mercuric chloride is subjected to heat, there results a further drop in resistance which remains constant through repeated centrifugings and resuspensions in Ringer solution.

The data presented in this paper show that in interpreting the results of resistance measurements the essential difference between the permeability of cells to ions moving under the influence of an *osmotic* gradient, and their permeability to ions moving under the influence of a *potential*

gradient must be fully recognised. Measurements of resistance give valuable information as to the osmotic changes occurring in the cell-solution system, but there is no indication that the permeability of the cell to ions moving under the influence of an electric current is in any way affected by the phenomenon of death.

13. *A Modification of the Brown Apparatus for the Determination of Colorimetric pH.* WILLIAM H. WRIGHT AND H. G. HARDING, College of Agriculture, University of Wisconsin.

An arrangement of the pH scale on the glass background according to the range of this indicator makes a more convenient set and permits comparison and cross checking of indicators. Clark and Lubs buffer solutions sterilized intermittently at 100°C. have been found to be entirely satisfactory and retain their accuracy.

14. *Limiting Factors in the Lactic Fermentation.* L. A. ROGERS AND E. O. WHITTIER, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

Cultures of *Strept. lactis* increase to a level which is constant for the same culture under fixed conditions. At this level there is no appreciable increase or death of cells but fermentation continues for some hours.

This level has a direct relation to the buffer content of the medium, is higher in milk than in broth, higher in milk or broth maintained at a constant pH, and still higher in media held at a constant pH and aerated. Holding the pH constant prolongs the fermentation but it finally ceases. Reducing the crowding effect by filtering and returning filtrate does not prolong multiplication or fermentation. The effect of concentration of undissociated lactic acid in stopping acid production is found to vary somewhat with other factors as indicated by earlier investigators. In an aerated milk culture the reduction potential is raised from approximately − 0.2 volt to + 0.2 volt. The oxidation-reduction level quite probably affects the population level.

15. *Bacterial Metabolism. The Influence of Phosphate Buffer in Sugar-Free and in Glucose-Containing Media.* CHAS. A. SLANETZ AND LEO F. RETTGER, Laboratory of General Bacteriology, Yale University.

Metabolism experiments were conducted on the following 22 organisms: *B. subtilis* (2 strains), *B. cereus*, *B. megatherium*, *B. anthracis*,

Bact. coli (2 strains), *Bact. typhosum* (2 strains), *Bact. paratyphosum* A and B (2 strains each), *Ps. pyocyanea, E. prodigiosus, Bact. bronchisepticus, Bact. pullorum* (2 strains), *Proteus vulgaris, Staph. aureus, C. sporogenes* and *C. Welchii.*

The studies were concerned with changes effected in amino acid nitrogen, non-protein nitrogen, ammonia nitrogen, biuret, H-ion concentration, glucose, and turbidity in buffered and in unbuffered plain and glucose broth.

The presence of balanced phosphate (1 per cent or M/7) in carbohydrate-free and in glucose-containing peptone had an accelerating effect on bacterial metabolism. This influence is directly associated with the maintenance of suitable H-ion concentration.

Very marked differences in nitrogen metabolism were observed between different types or groups of organisms, and even between species of the same genus, as for example, *B. cereus* and *B. megatherium.*

The "proteolytic" activity of some organisms may be inhibited or greatly retarded by the presence of fermentable sugar in the medium. However, this inhibition is not due directly to the sugar itself, but to the accumulation of products of carbohydrate metabolism, the organic acids.

When favorable environmental conditions are maintained, as, for example, by phosphate buffer, the type and degree of nitrogen metabolism exhibited by an organism in glucose broth may approximate those displayed by it in plain peptone broth.

Definite, though usually small, amounts of amino acids and ammonia are utilized by bacteria during the period of active growth. A greater decrease in amino and ammonia nitrogen generally accompanies the more luxuriant growths noted in buffered glucose-containing media than in the unbuffered and in the carbohydrate-free media during the period of rapid multiplication.

When *B. cereus* is grown under conditions of greatly reduced oxygen supply ammonia formation is very slight, but the changes in amino acid nitrogen compare favorably with those brought about under fully aerobic conditions.

16. Studies on the Metabolism of the Bact. Abortus-Melitensis Bronchisepticum-Alcaligines Group. 1. Nitrogen Metabolism. JAMES G. MCALPINE AND CHARLES A. SLANETZ, Storrs Agricultural Experiment Station, Conn.

Eight strains of *Bact. abortus*, five of bovine and three of porcine origin,

five strains of *Bact. melitensis* from various sources, two strains of *Bact. bronchisepticum,* and two strains of *Bact. alcaligines* were employed in this study. The organisms were grown in 1 per cent Fairchild's peptone broth, both with and without the addition of 1 per cent glucose. Duplicate sets were grown in the presence of 10 per cent carbon dioxide and in jars containing ordinary air. After incubation at 37°C. for two, six, nine and fourteen days, the broth cultures were analyzed for free ammonia, for amino nitrogen by the Van Slyke and Sörenson methods, for non-protein nitrogen and for the utilization of glucose by the Benedict method. The rate of growth was measured by the McFarland nephelometer and hydrogen-ion concentrations were determined by the colorimetric method of Clark.

The results of these preliminary experiments are briefly given as follows: Carbon dioxide accelerated the growth of the bovine strains of *Bact. abortus,* even though they had become accustomed to aerophilic conditions by long continued culture on artificial media. There was no growth-stimulation of the porcine strains by added carbon dioxide. The growth of *Bact. melitensis, Bact. alcaligines* and *Bact. bronchisepticum* was markedly retarded by the addition of 10 per cent carbon dioxide.

Hydrogen-ion determinations showed constant increasing pH values for *Bact. alcaligines, Bact. bronchisepticum* and bovine strains of *Bact. abortus,* even in the glucose-containing medium. In glucose broth, *Bact. melitensis* and the porcine strains of *Bact. abortus* constantly exhibited pH values approximating, or lower than those of the controls. This may be accounted for by the apparent utilization by *Bact. melitensis* and the porcine strains of *Bact. abortus* of small quantities of glucose as determined by the Benedict method. This apparent utilization was not so marked in the bovine strains of *Bact. abortus,* in the cultures of *Bact. alcaligines* or in those of *Bact. bronchiseptcum.* It has been assumed that certain nitrogen fractions tend to give high values in the Benedict test, and it is possible that the utilization of these substances by the bacteria in question would result in lower sugar values.

Bact. alcaligines, Bact. bronchisepticum and *Bact. abortus* of bovine origin showed a steady increase in free ammonia in the glucose broth during the fourteen days incubation. On the other hand, the porcine strains of *Bact. abortus* exhibited little or no change in free ammonia content. In the cultures of *Bact. melitensis* there could be demonstrated relatively little free ammonia before the ninth day.

There was comparatively little change in the amino-nitrogen content of the cultures of *Bact. melitensis* and the swine strains of *Bact. abortus*. The bovine strains of *Bact. abortus* utilized considerable amounts of amino-nitrogen during the fourteen-day period of observation. This utilization also was very marked in the cultures of *Bact. alcaligines* and *Bact. bronchisepticum.*

From these preliminary experiments there appears to be a decided difference in the metabolic activities of *Bact. melitensis* and *Bact. abortus* of bovine origin, at least in the strains used in this study. Cultures of *Bact. abortus* from porcine sources resembled more closely *Bact. melitensis* than the bovine strains of *Bact. abortus.*

17. A Biochemical Method of Differentiating Brucella abortus from Brucella melitensis-paramelitensis. I. FOREST HUDDLESON AND ELIZABETH ABELL, Bacteriological Section, Michigan Agricultural Experiment Station.

It has been found that *Brucella abortus* in its growth activity on a suitable medium, under aerobic incubation causes the liberation of hydrogen sulphide gas while *Brucella melitensis* or *Brucella paramelitensis* does not. The gas may be detected by means of lead acetate paper.

In this study 92 strains of *Br. abortus*, 30 strains of *Br. melitensis* and 10 strains of *Br. paramelitensis* were employed. All of the strains were isolated either in Europe or in this country from bovine, swine, human, caprine and equine sources.

The hydrogen sulphide test so far has agreed with the agglutination absorption test in placing the strains in the abortus or melitensis group. The hydrogen sulphide test offers a rapid procedure for the grouping of newly isolated strains.

18. Studies in Bacterial Metabolism: On the Selective Utilization of Amino Acids by Bacteria. E. LEE TREECE, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

The utilization of a number of amino acids by members of the intestinal group of bacteria was studied by means of growth rates and by a comparison with the utilization of cystine in a synthetic medium. Hydrogen sulphide production was used as an index of the utilization of the cystine by the organism.

In this way it is shown that when two amino acids are present, one may be used in preference to the other. Such sparing of cystine serves

to explain the delayed hydrogen sulphide production from peptone by certain bacteria of this group.

19. *A Blood-Clot Digest Medium for Cultivation of Hemophilic and Other Bacteria.* R. S. SPRAY, School of Medicine, West Virginia University, Morgantown, W. Va.

It is believed that the medium herein described preserves the valuable features of chocolate agar, while it also eliminates the tedious details of the aseptic preparation required for routine chocolate agar.

After removing serum from blood clot for preparation of Loeffler's medium the clot is drained and comminuted by passing through wire gauze. It is then boiled and again finely divided. One liter of the semi-fluid material is placed in a 2-liter flask; 20 grams anhydrous sodium carbonate; 5 grams trypsin; and 15 to 20 cc. chloroform added.

The fluid is incubated for fifteen days, with a second addition of 3 grams trypsin on the tenth day. Chloroform may be added on the fifth and tenth days to prevent bacterial decomposition.

By the fifteenth day digestion is practically complete. The fluid is then rendered strongly acid with HCl (50 to 75 cc.) and steamed in water-bath to drive off chloroform, then titrated with normal NaOH to reaction pH 7.4 to 7.5; tubed, and autoclaved. If tubes are paraffined the medium keeps indefinitely.

This blood-clot digest, thus prepared similarly to Wolf's casein-digest, may be added in from 5 to 10 per cent to melted North's, or other agar, tubed and sterilized like plain agar. On the resulting medium *H. influenzae*, *Pneumococcus*, and *Streptococcus* grow abundantly. When sodium oleate is added, as in the Avery medium, *H. influenzae* grows very vigorously.

If the blood-clot digest is filtered through cotton a clear filtrate is obtained which, added to agar gives a clear medium similar to hemolyzed blood agar, upon which *H. influenzae* colonies are quite characteristic.

20. *Bacteriophage Active against a Thermophilic Bacillus.* STEWART A. KOSER, Hygienic Laboratory, University of Michigan and the Department of Bacteriology, University of Illinois.

A lytic principle, or bacteriophage of d'Herelle, active against a thermophilic organism was obtained from sewage polluted river water The organism in question is a slender spore-forming rod. Its optimum growth temperature appeared to be from about 45° to 52° C. and development at this point was more rapid than at 37°C. The maximum

temperature was 58° to 60°C. At 20° to 25° the culture developed slowly, requiring several days to produce scanty growth on agar slants. A lytic filtrate was developed by the usual method of alternate feeding and filtration at 37°. After development at this temperature it was found to be active against the homologous culture at various points from room temperature (20° to 25°C.) up to about 58°C., with very pronounced and vigorous action at 50 to 52°.

The filtrate fulfilled the commonly accepted tests for demonstration of the bacteriophage. It caused both inhibition and lysis of the culture, was transmissible in series, and in appropriate dilutions formed plaques of lysis on agar slants previously inoculated with the culture. At 50° to 52°C. the plaques were larger than at 37°C. Tests for inactivation showed that this "thermophilic bacteriophage" survived heating at 70°C. for thirty minutes but was destroyed at 75°C. Thus, in spite of its activity at higher temperatures, its heat resistance is about the same as that reported for the usual coli, typhoid or dysentery bacteriophage.

21. An Adaptation of the Chambers Microdissection Apparatus for the Isolation of Single Bacterial Cells. WILLIAM H. WRIGHT AND ELIZABETH F. MCCOY, College of Agriculture, University of Wisconsin.

The double Chambers apparatus as made by Leitz has been found to be very well adapted to the isolation of single bacterial cells. The 13 mm. chamber made by Leitz is easily converted into a moist chamber where drops of sterile solutions as small as 5 to 10 micra can be held without evaporation for over an hour. Single cells are isolated in such a small drop with one pipette and transferred to sterile culture media with the other.

Two types of micropipettes have been found most useful. One is a 45° type for making the small drops and one a 90° type for removing single cells. Neither should have a tip bore of less than 5 or more than 10 micra for bacteria and 25 to 30 micra for yeasts.

Advantages of the technique are: (1) Certainty of *single cell* cultures. (2) Ease of manipulation—six isolations in thirty minutes under best conditions. Average is about 3 per hour. (3) The technique is entirely practical for the isolation of pure cultures where selective cultural methods often fail. (4) Single cell isolations have given growth with about 90 to 100 per cent of young yeast cells, 25 to 30 per cent with aerobic bacteria and 1 per cent with anaerobic bacteria.

SYMPOSIUM ON "FILTERABLE VIRUSES"

1. *Filterable Viruses—A Critical Review.* T. M. Rivers, Rockefeller Institute for Medical Research, New York City.

Throughout this discussion the term "filterable viruses" is used in a non-committal way to designate active transmissible agents which are capable of producing pathological conditions in bacteria, plants, insects, fish, birds, and mammals, and which by general consent are limited for the moment to the number of active agents connected with the diseases listed in the table. The arrangement of the diseases is for convenience of discussion and carries no classificatory significance. In the first place, filterability of the etiological agents does not sharply delimit this group of diseases, as it is well known that the viruses share this characteristic with certain small bacteria and vibrios, and also with some spirochetes and protozoa. Furthermore, in regard to the etiological agents of some diseases, within the group, either no filtration experiments have been recorded or there is much discussion as to whether they are filterable. In the past all attempts to classify these diseases have been unsuccessful and there is every reason to believe that such attempts are still premature. The diseases listed form a heterogeneous group. In fact they exhibit so many differences that a discussion of the filterable viruses almost amounts to a separate discussion of each disease. Such a state of affairs is due to the fact that the filterable virus group has been used to a considerable extent as a dump heap for infectious diseases of unknown etiology. Therefore, it is not unlikely that some of them will be shown to be caused by small bacteria or protozoa. When this occurs such diseases should be removed from the filterable virus group and given their correct position in the classification of diseases.

Epidemiology. Epidemiological problems presented by the virus diseases in regard to regional distribution, seasonal variation, host susceptibility, and virulence are similar to the problems found in connection with other infections.

Immunity. It is doubtful, with a few possible exceptions, whether injection of a virus completely inactivated leads to a protection against the same virus in an active state. Diseases produced by the filterable viruses, if recovered from, usually lead to a lasting immunity. In this respect virus diseases differ from those caused by ordinary bacteria. This is not universally true, however, since one attack of typhoid fever produces a fairly lasting immunity. No satisfactory explanation has

been found for the enduring immunity. Whatever the true cause of the phenomenon may be, it is not unlikely that it is closely related in some way to the peculiar parasitism exhibited by the viruses.

Prevention of virus diseases. The prevention of virus diseases depends upon the protection of susceptibles from exposure or upon their immunization by means of vaccination with attenuated or modified viruses.

Filterability. Since the discovery of the first filterable virus in 1892, it has been determined by means of different kinds of filters that many diseases are caused by active agents smaller than ordinary bacteria. Some of them are presumably much smaller and are most likely optically immeasurable. Others, however, do not seem to be so small and concerning their filterability there is much discussion. Methods of filtration are crude and inaccurate. The most one can say concerning the viruses is that under given experimental conditions they either pass or do not pass through certain filters. The failure to pass a filter, however, is certainly not determined in every instance by the size of the virus. Filtrates from uncontrolled, and even from well controlled, sources may contain more than one active agent, some of which may be cultivated on artificial media. All investigators should be extremely careful in working with filtrates not to be misled by their findings and ascribe to an active agent an etiological rôle in a disease with which it has nothing more than an accidental connection.

Size. The size and weight of molecules of crystalline egg albumen and crystalline hemoglobin are not agreed upon. Consequently, what hope is there at present of ascertaining the size of viruses which have not been obtained in a pure state?

Cultivation. No worker has proved that any of the etiological agents of the diseases in the table down to mumps can be cultivated in the absence of living cells. A satisfactory explanation of the difficulty experienced in cultivating the viruses on artificial media is not easily found. Their small size alone should not necessarily make them insusceptible to cultivation. Nor does it seem to be a question of delicacy or sensitiveness, because many of them are extremely resistant to chemical and physical agents. Furthermore, no virus has been found multiplying free in nature. The viruses appear to be obligate parasites in the sense that their reproduction is dependent upon living cells.

Cell types in relation to virus reproduction. In view of the fact that most viruses multiply only in the presence of living cells, it is advisable to ascertain what kinds of living cells promote their reproduction best,

and what effect upon the cells is induced by the reproduction. A remarkable species specificity is exhibited by many viruses. Frequently young cells seem essential for the activity of the viruses. Some viruses show an affinity for cells of certain tissues and apparently can neither multiply nor produce signs of disease unless they come into close relation with these cells. It is not known whether the viruses multiply intra- or extracellularly. Nevertheless, they have a profound influence upon cells and cause remarkable changes within them. This influence most likely accounts for the fact that in lesions caused by many viruses intracellular changes assume appearances characteristic enough to be spoken of as inclusion bodies. In this respect many virus diseases differ from those caused by ordinary bacteria.

Inclusion bodies. Inclusions may occur in the cytoplasm, in the nucleus, or in the cytoplasm and nucleus. Various ideas are held concerning them. Some investigators consider them merely as products of degeneration, but others believe that they are the virus itself, while yet others think of them as virus surrounded by a mantle of altered cellular material. As yet their nature has not been definitely determined. Nevertheless, in spite of the ignorance concerning their nature, inclusion bodies have held and will continue to hold an important position in the study of this group of diseases. Many attempts to produce significant inclusions by artificial means have been unsuccessful. Therefore, under properly controlled conditions the presence of inclusions, accepted as significant, is undoubtedly in the majority of instances indicative of the presence of a virus in the immediate vicinity.

Characteristics of filterable viruses. A wide range in the degree of resistance to physical and chemical agents is exhibited by the viruses. The question of the organized or corpuscular nature of viruses has not been satisfactorily settled. Bacteriophage, rabies virus, and herpes virus, in the absence of living cells, do not respire enough to be detected. Phenomena have been observed that cause one to ask whether viruses can mutate. Whether it is correct to speak of these phenomena as examples of mutation is not known. In any event, when viruses are adapted to alien hosts, their characteristics are frequently altered, as well as those of the diseases produced by them. It is impossible to say at present whether the viruses are animate or inanimate. In regard to the filterable viruses, it can be said that they exhibit, when compared one with another, a diversity of characteristics equal to, if not greater than, that exhibited by ordinary bacteria and other known forms of life. Consequently, one should not generalize when dealing with this remarkably heterogeneous group of active agents.

Filterable Virus Diseases

Bacteriophage

Mosaic diseases of plants
(infectious chlorosis)

Sacbrood
Wilt of European nun moth
Wilt of gypsy moth caterpillar
Jaundice of silk worms

Epizootic of guinea pigs
Hog cholera
Cattle plague (Rinderpest)
Pernicious anemia of horses
Virus III infection of rabbits
Foot-and-mouth disease
1 Type A
2 Type O
Vesicular stomatitis of horses
Paravaccinia (No report on filtration)

1 Swine-pox
Goat-pox
Sheep-pox
Horse-pox
2 Cow-pox
Smallpox
3 Varioloid
Alastrim
4 Chicken-pox
5 Herpes zoster (No reports on filtration)
Symptomatic herpes
6 Encephalitis
1 Lethargic
2 Following vaccination 9
3 Japanese 1924
4 Koritschoner
7 5 Australian X disease 8
Poliomyelitis
Rabies
Borna's disease
Fowl plague and plague of blackbirds
Guinea pig paralysis
Distemper of dogs

Trachoma and inclusion blenorrhea
Infectious papular stomatitis of cattle
Molluscum contagiosum
Warts
Contagious epithelioma (fowl-pox)
1 Chickens
2 Pigeons
Infectious myxomatosis of rabbits
Rous sarcoma of chickens
Leukemia of chickens
Lymphocystic disease of fish
Epithelioma of fish
Carp-pox
(No reports on filtration)

Mumps (According to Kermorgant, a spirochetal disease)
Agalactia (According to Bridré, a bacterial disease)
Salivary gland disease of guinea pigs

Measles (rubeola)
German measles (rubella) (No report on filtration)

Grippe (influenza) (According to Olitsky and Gates a bacterial disease)
Common colds

A Insect-borne

B
Nairobi disease of sheep
Catarrhal fever (blue tongue) of sheep
African horse sickness
Pappataci fever
Dengue fever
Yellow fever (According to Noguchi a spirochetal disease)

C Rickettsia diseases
Typhus fever
Trench fever
Rocky Mountain spotted fever
Heartwater disease
Flood fever of Japan

D Bacterial diseases
Oroya fever and verruga peruviana
Pleuropneumonia
Avian diphtheria
Scarlet fever

Majority of the diseases which have been placed in the filterable virus group by different workers

Identity of the epitheliotropic and neurotropic viruses. In the table, the diseases from swine-pox through rabies have been arranged and bracketed in a way that quickly shows the relation claimed by different workers to exist between members of the group. Many of the viruses may be closely related or some may have evolved from a common ancestor, yet it is unlikely that all claims in regard to the identity of these viruses are correct. More experiments are needed to settle some of the debated questions.

Summary. In the majority of the virus diseases there exists a very close relation between the etiological agents and the cells of the hosts. This peculiar type of parasitism, if you choose, may account for the fact that some of the diseases show a striking species specificity, that the viruses have evaded cultivation on simple media, that characteristic or specific pathological changes are frequently observed in cells affected by viruses, and, finally, that a host once recovered from a virus disease usually possesses a lasting immunity.

2. *The Nature and Significance of Cellular Inclusion Bodies in Diseases Due to Filterable Viruses.* E. V. COWDRY, Rockefeller Institute for Medical Research, New York City.

From the cytological point of view the inclusion bodies which are developed in rabies, herpes, vaccinia, chicken-pox, and several other diseases listed in this rather ill-defined group are of great interest. They represent reactions on the part of the cells to influences of which very little is known. These reactions are often highly specific and absolutely without counterpart, to the best of our knowledge, in any other physiological or pathological conditions.

The inclusions themselves, occurring as they do not only in man and many vertebrates, but also in certain insects and plants, are characterized by great diversity. For this reason generalizations are difficult to make, and are often stultified by the number of qualifications and exceptions which must be noted. Yet, to attempt to envisage the series of reactions, as Findlay and Ludford (1926) have done, seems worth while. Nuclear inclusions, as one would expect, are less frequently associated with disease than cytoplasmic ones, and are also less varied in character, because the nucleus occupies a protected position, being, in large measure, sheltered from the environment by its investment of cytoplasm, and because its chemical constitution is more restricted. We cannot doubt that the incidence of inclusions, both nuclear and cytoplasmic, is in reality a question of the permeability of a peculiar group of

substances, some of them remarkably stable in character. The inclusions, with but few exceptions, exhibit the property of growth by accretion. In some cases they are formed largely, or in part, by a kind of overproduction of material, traces of which may be observed antecedent to the action of the viruses. Very obviously the *Rickettsiae*, which have sometimes been included in this category, belong elsewhere. Our information regarding these specific inclusions is in a sense superficial. No single type of inclusion has been studied with the concentration that has been lavished upon a zymogen granule of the pancreas, for example. Many methods of cytological study devised within the past decade have apparently never been applied. The attention of specialists has but rarely been invoked. Biochemists interested in cellular physiology have much to offer. Great refinements in cytological technique have been perfected in the study of the nucleus, and yet investigators who have devoted their lives to a better understanding of the nucleus have never felt impelled to study these truly remarkable nuclear inclusions. The inclusions in plants have been studied by botanists, those in insects by entomologists, and those in higher forms by physicians and pathologists. Recognition that the problem is common to all makes for progress.

3. Recent Research on Foot-and-Mouth Disease, with Special Reference to the Work of the American Commission. HARRY W. SCHOENING, Bureau of Animal Industry, Washington, D. C.

A brief review of the recent research work on foot-and-mouth disease with special reference to the work of the Commission appointed by the United States Department of Agriculture to study foot-and-mouth disease is given. Active work was done by the Commission at Strasbourg and Alfort, France, in addition to an epidemiological study of the disease in various countries.

The disease was transferred to guinea pigs and carried through 261 passages. The virus after numerous guinea pig passages was still highly active for larger animals. Attempts at cultivation of the virus were unsuccessful. The work of Frosch and Dahmen could not be confirmed. A study of factors enhancing survival of the virus in artificial media indicated the following to be essential: A hydrogen ion concentration of pH = 7.5 or 7.6, temperature less than 37°C.; the optimum being room temperature, while anaerobic atmospheric conditions were found to be more favorable than aerobic. The best suited medium was of the simplest kind and of a semi-solid nature; 0.25 per cent agar or 10 per cent

gelatin. At room temperature the virus in such media remained alive for more than 69 days but less than 100. No multiplication of the virus *in vitro* was observed.

Under field conditions, in hay or garden soil, the virus was found active for at least twenty-five to thirty days.

By means of cataphoresis the electric charge of the virus was found to be positive, the isoelectric point being pH = about 8. The virus was regularly filterable through Seitz asbestos discs and Berkefeld V candles, and Chamberland bougies L-1 to L-5. Some adsorption of the virus occurred in Berkefeld N and the Chamberland L-7 and L-9 bougies, while in the L-11 bougie it was completely retained. The virus being electropositive and the filters electronegative, adsorption occurred in the denser filters due to oppositely charged materials. When the charge of the virus was changed to negative it readily passed through the Chamberland L-11 bougie. Positive filtrates were also obtained with the most porous of Bechold's ultra-filters.

The virus was found active in dilutions as high as 1:10 million. The size of the virus has been delimited by molecular filtrations to be between $20\mu\mu$ and $100\mu\mu$ in diameter, indicating that it is particulate and not of a fluid character.

The abnormal resistance of the virus to such antiseptics as alcohol, acetone, bichloride of mercury and lysol is explained by the precipitation of protein materials by these chemicals, as a result of which the virus is protected by the coagulum. When this precipitation is prevented, 60 per cent alcohol, for example, will kill the virus in less than 1 minute. Sodium hydrate or antiformin, which cause no precipitation, readily destroy the virus.

It was found by experiments on guinea pigs, cattle and hogs that at least two types of foot-and-mouth disease virus exist, thus confirming the work of Vallee and Carré. The disease produced by these viruses is indistinguishable clinically, but they do not cross immunize.

Under rigid experimental conditions the presence of a carrier could not be demonstrated in 20 cattle recovered from foot-and-mouth disease and especially selected by Swiss officials as being likely carriers of the virus. Field evidence on this point is strong, however, although the percentage of animals acting as carriers is believed to be small.

The hoofs of 22 cattle and 1 hog were examined at post-mortem twenty days to six months after the onset of the disease. Scrapings were made of sawed sections and guinea pigs inoculated. One positive result was obtained in a cow thirty-four days after infection.

The earthworm could not be implicated as a carrier.

Cattle were found to contain active virus in the saliva before any lesions of the disease were observable. Tests on guinea pigs showed materials taken from infected cattle to be infectious no longer than seven days after the first appearance of symptoms. In most cases, however, such materials were inactive after four days.

Immunity follows an attack of foot-and-mouth disease and is of two types, local or tissue and general or blood. The tissue immunity is the first to disappear and as a rule may be said to last at least from four to six months, and the general immunity from one to two years, although much shorter and longer periods have been recorded. Limited attempts at artificial immunization were unsuccessful.

By means of guinea pig tests antibodies can be demonstrated in immune and hyperimmune serums.

Hyperimmune serum from cattle showed about the same potency as so-called reconvalescent serum. By repeated injections of virus into rabbits a more potent serum was obtained. Normal horse serum, or serum from a horse repeatedly injected with virus, showed no protective properties. Horses were resistant to infection with both types of foot-and-mouth disease virus. Complement-fixation tests were uniformly negative.

A comparative study was made between vesicular stomatitis and foot-and-mouth disease. A marked similarity was found in the two diseases, the distinguishing features being a lack of cross immunity and the susceptibility of the horse to vesicular stomatitis and its resistance to foot-and-mouth disease.

4. *Some Characteristics of Virus Diseases of Plants.* L. O. KUNKEL, Boyce Thompson Institute for Plant Research, Yonkers, N. Y.

Virus diseases of plants are systemic maladies producing in most cases marked chlorosis. They are caused by filter passing agents. They produce two general types of disease. In the first type, chlorosis is noticeable in all chlorophyll-bearing tissues and diseased plants are yellowed. In the second type, chlorosis is confined to certain areas irregularly distributed in the green tissues so as to produce a color pattern characteristic of the so-called mosaic diseases. Both types cause stunting, upright habit of growth, and abnormal production of secondary shoots. They seldom kill plants. Some of these diseases are highly infectious, some are transferred with considerable difficulty, and a few have never been transmitted except by grafting. Many of

the virus diseases of plants are spread by sucking insects. Some show an incubation period in the carrier insects. They are most readily transmitted to rapidly growing plants. Plastic cell inclusions are associated with several different mosaic diseases. These intracellular bodies are present in chlorotic tissues but are not found in the normal green tissues of diseased plants.

5. *Studies on Vaccinia Virus.* E. H. OPPENHEIMER, Department of Pathology, Johns Hopkins Medical School, Baltimore, Md.

By means of differential centrifugation, it was determined that the specific gravity of vaccinia virus lies between 1.12 and 1.13. In material obtained by this treatment of ordinary vaccinia lymph, myriads of small granules in masses, pairs and chains were seen by darkfield illumination. The infectivity of the material, as determined by the production of vaccinia lesions on the scarified cornea of the rabbit, was always associated with the presence of these granules. Purification of the virus was accomplished by centrifugation and washing in a fluid slightly heavier than the virus.

The virus could not be filtered through any grade of Berkefeld filters. When filtered through paper, wool or chamois, infective filtrates always contained myriads of these granules.

The granules, then, seemed to form an important part of the infective material. To determine the relative importance of the granules and their suspending fluid, Dr. Cracium and I isolated these materials by differential centrifugalization and tried to make each multiply in connection with growing epithelium as Steinhardt and Lambert had done with the whole virus.

We removed 0.1 cc. of the top layer of the whole virus after centrifugalization and added it to 0.9 cc. of sterile Locke solution. The granules were washed thoroughly by pouring off the supernatant fluid and adding fresh Locke solution. This was mixed, again centrifugalized, and the precipitated granules washed twice again in a similar fashion. Before inoculating the granules into the tissue cultures, they were always diluted to their original concentration in sterile Locke solution. The first supernatant fluid was used as the control material. This was not diluted as it already contained Locke solution. The most satisfactory tissue for culture was embryonic rabbit cornea, grown in homologous heparin plasma. A drop of the diluted washed granules or control material was added to a bit of cornea with a drop of plasma, sealed over deep hollow ground glass slides and incubated at 38 C. Every

three to seven days, the cultures were washed in Locke solution and put side by side with a new piece of cornea and fresh plasma. In the intervals, that is, every two or three days, the fluid part of the cultures was pipetted off and a fresh drop of plasma added. About every eight days the presence of the virus was tested by inoculation on a scarified rabbit's cornea. The washed granules and control material used for the series were similarly tested beforehand, the granules always, and the control material never, giving the vaccinia lesion. Whereas the fluid part of the vaccinia virus will not produce a lesion even after being in contact with growing epithelial cultures, the granules will keep alive for seventy-one days and through 9 transfers. They not only live but actually increase in potency. It was shown further that, as with the virus, living tissue is necessary to the life of the granules.

Another variation was tried to see whether the granules enter the growing cell, or whether they merely remain alive in the plasma and will disappear if the original piece of tissue is removed after being in contact with the new piece for a few days. The cultures were made as above and transferred to a new bit of cornea after seven days. At this time a camera lucida sketch of the culture was made—the original inoculated piece of tissue labeled "A" and the fresh piece of cornea to which no granules were ever added, labeled "B." After seven more days, both "A" and "B" were removed, washed in Locke solution separately, and each added to a new medium of cornea and plasma, thus giving rise to an "A" and "B" subdivision of the same series. After a sufficient length of time, both of these were inoculated separately to test for the presence of the virus. Both series contained live virus, the "B" as well as the "A," showing that it not only is located inside the cell, but that it must, in part, leave the original site of inoculation for the more favorable fresher tissue. The "B" cultures always had a shorter incubation period after inoculation and gave a larger vaccinia lesion than did the "A" cultures.

We have from these studies no morphological proof of an increase in the number of granules, since they cannot readily be distinguished from other granules normally seen in tissue cultures. But the experiments differ from others which have been concerned with the cultivation of the virus of vaccinia in that the granules have been washed free from all adhering fluid and then cultivated in increasing potency, while the fluid in which they were formerly suspended shows no power to infect the rabbit's cornea even after cultivation. They show, at least, that the power of the vaccine to infect an animal is inherent in the granules even though further analysis of their nature must be deferred.

6. *The Mouse as a Test Object for the Herpetic Virus.* CHARLES E. SIMON, School of Hygiene, Johns Hopkins University, Baltimore, Md. (No Abstract.)

COMPARATIVE PATHOLOGY AND IMMUNOLOGY

AND

SYMPOSIUM ON "FILTERABLE VIRUSES" (*Continued*)

1. *A Study of the So-Called "Specific Factor" (Gye) of Chicken Sarcoma (Rous).* JOHN A. KOLMER, MALCOLM J. HARKINS AND JAY F. SCHAMBERG, Research Institute of Cutaneous Medicine, Philadelphia, Pa.

A brief résumé of Gye's hypothesis of the nature of malignant growths is given and important technical factors in relation to the preparation of the so-called "specific factor" are described. The results of studies in chicken sarcoma failed to corroborate Gye's work and hypothesis. Evidence indicates that the so-called "specific factor" contains living virus. The virus of sarcoma was not successfully cultivated.

2. *The Virus Problem in Transplantable Tumors.* J. HOWARD MUELLER, Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Mass.

Attempts to repeat the experiments published in 1925 by Gye, and from which radically new conclusions as to the etiology of tumors were drawn, have lead to a variety of different results, none of which completely confirm Gye and many of which are totally different. This discussion will deal with his experiments on the Rous chicken sarcoma, from which he prepared a specific factor and a virus, each by itself non-infectious, together causing a tumor in chickens.

His conclusions have been criticized by Harkins, Schamberg, Kolmer and Kast, who found the specific factor controls always infectious when tumors were produced in the mixtures with virus. Our own results, and also those of Cutler, are similar to these to the extent that tumors have never been consistently obtained in a mixture of the two factors unless one or the other also gave positive controls.

It is evident that whatever one may believe to be the interpretation of the experiments published by Gye, no definite conclusions can be reached until the experiments themselves can be repeated by various workers with uniform results. The discordant findings thus far re-

ported must be explained. Three varying factors are involved: (1) the tumor strain; (2) the specific factor; and (3) the virus. None of these is of a nature which lends itself to control by simple means, yet obviously each must be controlled before Gye's experimental results can be justly contradicted.

Considerable importance must be placed on the type of controls carried out. Thus we know that there is a marked variation in susceptibility on the part of chickens to injections of the attenuated Rous agent. Our own results demonstrate clearly that this may be extended even to local variations on the same bird, two injections of the same material into each of a series of chickens not infrequently leading to the development of one tumor instead of two. If two factors are involved, and controlled at different sites on the same animal, may one be carried by the blood to the site of the second and produce a false positive? Only by using a fairly large number of chickens for each experiment can one expect to obtain results beyond criticism, and within such a series the findings must be consistent. An occasional result of the type expected, with many others negative, must be held to be fortuitous.

If one may venture to draw conclusions from all the work so far made public, it must be that Gye's experiments have neither been confirmed nor disproved, and further, that it will perhaps be impossible for an individual worker to refute them in a final way because of the uncontrollable factors above referred to. Because of this negative results cannot, by present methods, signify final refutation. The matter must rest on this basis until Gye himself or someone with sufficient faith in the results of Gye, or in those of Murphy and of Flu have discovered means of rendering positive results less dependent upon uncontrollable accident.

3. Virulent Streptococci and Spore Forming Rods Cultivated from So-called Herpetic and Encephalitis Viruses. ALICE C. EVANS, Hygienic Laboratory, Washington, D. C.

Five strains of herpes virus and one strain of encephalitis virus received from various laboratories were available for study. By planting emulsions of virus in meat medium, cultures of *Streptococcus* and cultures of spore forming rods similar to those cultivated from several cases of epidemic encephalitis were obtained from all of the viruses, although no growth was obtained when ordinary methods of cultivation were employed. Cultures of these forms could also be obtained from filtrates of virus emulsion. Both forms of the organism were virulent for rabbits when inoculated intracerebrally.

4. Are Certain of the Ultraviruses, So-called, Filtrable Stages in the Life Cycle of Ordinary Bacteria? RALPH R. MELLON, Highland Hospital, Rochester, N. Y.

In a study published in 1919 we showed conclusively that a filtrable phase of a coccus indistinguishable morphologically from a staphylococcus existed in the blood stream of a case of septicopyemia. This was apparently one of the earliest demonstrations that ordinary bacteria possessed filtrable phases, although Fontes in 1910 made the same claim for *B. tuberculosis* and in 1916 Almquist confirmed it for *B. typhosus.* Since the discovery of the bacteriophage the proposition has been repeatedly confirmed.

The fact that these filtrable phases of bacteria are usually not cultivable, and are frequently invisible, raises a point of fundamental importance in relation to the so-called filtrable viruses. May many of the latter be stages in the life history of some visible and commonly recognized microörganisms?

The question is particularly pertinent when the so-called virus disease is associated with a constant variety of visible microörganism. The conventional interpretation of the latter as an unrelated secondary invader may thus be seriously impugned. As examples I may mention only the association of *B. proteus* with the virus of typhus, the *B. suipestifer* with the virus of hog cholera, *Streptococcus viridans* with encephalitis lethargica.

The work to be presented today indicates that *B. fusiformis* and related organisms have a filtrable phase in their life history, uncultivable in vitro but not invisible. Moreover, under the conditions of the experiments it is clear that this phase had an infectivity in guinea pigs not possessed by the visible phase. The typical lesions and the accompanying bacterial flora have been reproduced in guinea pigs. The experiments open up considerations of obvious epidemiologic importance. They raise the question whether actual epidemic infections are initiated by the invisible and uncultivable phases of common and perhaps avirulent bacteria.

5. Experimental Immunization in Poliomyelitis. W. L. AYCOCK AND J. R. KAGAN, Department of Preventive Medicine and Hygiene, Harvard Medical School, Boston, Mass.

Killed virus of poliomyelitis possesses no immunizing power. Inmunization with virus treated with phenol or by drying is successful in only one-fourth of the animals used, while about the same proportion

of animals become infected from the subcutaneous injection of the "attenuated" virus. Subcutaneous injection of active virus also immunizes but causes infection in about the same proportion of animals as in the case of "attenuated" virus. It appears that virus subjected to phenol or to drying has little if any less infectivity than active virus when injected subcutaneously.

Active virus injected intracutaneously has caused infection in only 1 out of 12 animals. Of 11 monkeys receiving intracutaneous inoculations of active virus over periods of from thirty-three to one hundred and forty-seven days, 7 resisted from 1 to 3 intracerebral inoculations; 2 resisted 1, but were infected by a second intracerebral inoculation. In 1 of these the incubation period was prolonged. One of the vaccinated animals succumbed to a single intracerebral inoculation, but the incubation period was prolonged.

Blood sera of 8 vaccinated monkeys neutralized virus *in vitro*. Fifteen specimens of sera neutralized the virus while 2 specimens partially neutralized as evidenced by prolongation of incubation period. These sera were from animals whose sera had previously neutralized the virus.

In respect to the epidemiology of poliomyelitis these observations lend support to the idea that individuals may become immunized to the virus of poliomyelitis without manifestations of the disease and that the neutralization of virus by blood sera of persons not known to have passed through an attack of the disease is evidence of immunity resulting from the virus, and not a property of normal serum.

6. Diabetes Mellitus: An Experimental Study on the Etiology of the Disease. (Preliminary Report.) D. H. BERGEY, Department of Hygiene, University of Pennsylvania, Philadelphia, Pa.

Since the etiology of diabetes mellitus has not been traced to either bacterial or protozoal infection, it was believed that the steady increase in the death-rate indicated some definite toxic action and suggested the possibility that a filtrable virus may be the responsible agent. The question was approached by passing the urine of diabetics through Berkefeld filters and then inoculating rabbits by administering a dose of about 2 cc. of the filtrate into the blood stream. In about three weeks, glucose appeared in the urine of the rabbits.

On inoculating the filtered urine of diabetics into serum broth medium, there is apparent increase of infectivity since the inoculation of a small dose of the culture caused the appearance of glucose in the

urine in about a week. Subcultures are still infective after cultivation for 6 months.

The filtered urine of diabetic rabbits, when injected into the circulation of normal rabbits causes the appearance of glucose in the urine in about a week. The filtered urine of a diabetic patient sealed in glass ampoules for over 9 months is still infective for rabbits.

7. *Studies of Bacterial Allergy with the Pneumococcus.* HANS ZINSSER AND FRANCIS B. GRINNELL, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

Extreme forms of skin reactions are difficult to develop in guinea pigs by ordinary methods of sensitization with bacteria.

Injections of autolyzed pneumococci into a large series of guinea pigs, if the autolysates are properly prepared, give occasional violent reactions, with hemorrhage, necrosis and ulceration in about 45 per cent of the old animals, but in practically none of the young animals of 250 to 350 grams.

These reactions, at first regarded as primarlly toxic reactions, have been shown to be allergic by the following facts:

Animals that are negative upon first test can invariably be sensitized by repeated injections. Such sensitization can be removed by one or several injections of considerable amounts of the autolysate.

Sensitization can be obtained by a number of different methods, most of them following the ordinary laws of sensitization in general, but it is particularly noticeable that many guinea pigs can be rapidly sensitized by daily injections in from four to seven days.

The reactions are extraordinarily severe when fully developed, covering areas of an inch square or more, with sagging oedema toward the abdomen and central tissue destruction. They are more violent than the most severe tuberculin reactions obtainable in guinea pigs. They can be elicited, both as to sensitization and final test, by the products of autolysis and products of bile solution of centrifuged masses of pneumococci.

It is likely that the responsible antigen is an autolytic product and that bile solution is merely a hastening of autolysis, as suggested by Atkins. It may be, however, that this method of preparing the antigen is merely one of getting an unaltered antigen into solution.

It is suggested that if other bacteria could be similarly autolyzed, similar severe and rapid sensitization might be accomplished with all organisms. This is being studied.

It is also suggested that actual sensitization of the infected body occurs in this way because organisms that cannot be prepared easily *in vitro* in the manner in which this is possible with the pneumococcus are probably subjected to lytic change under the influence of the tissues in lesions. This is a clue to the understanding of why tuberculin, abortin, etc., reactions are best obtained in animals in whom living bacteria and tissue changes around the bacterial foci occur. It suggests an explanation for our success in sensitizing with dead tubercle bacilli when tubercles are formed around them and would explain our former results in which tissue action upon tubercle bacilli and tuberculin seemed to be involved in tuberculin reactions.

The speed of sensitization and the suddeness of desensitization, coupled with the well-known and massive autolysis of pneumococci in the human lung during pneumonia, suggests a relationship between sensitization to the autolytic substances and the toxemias and sudden critical desensitization of the patient in pneumonia.

8. *Genetic Studies on Resistance to Bacterial Infection.* E. ROBERTS AND L. E. CARD, University of Illinois.

In 1924 several hundred chicks were inoculated by feeding through pipettes a pure culture of *Salmonella pullora* the causative organism of bacillary white diarrhea, one of the most fatal diseases which affect young chicks. A few were found which apparently resisted infection and these constituted stock for future breeding.

Chicks from this "resistant stock" in 1925 gave a survival value more than twice as great as that in control chicks In 1926, 55 per cent of the progeny of the resistant stock survived inoculation while only 10.1 per cent of the control stock survived. Among progeny of hens selected for one generation 48 per cent survived while among the progeny of those selected for two generations 60 per cent survived.

In the fall of 1926 those individuals which in the Spring tests had above 66 per cent survival among their offspring, and those below 45 per cent were put into separate lots. The chicks from the "high lot" gave a survival value of 80.7 per cent while those from the "low lot" gave 61.0 per cent.

The mortality of chicks from infected hens was found to be no greater than the mortality of chicks from non-infected hens.

9. *Observations on the Epidemiology of Lobar Pneumonia.* M. A. JACOBSON, Department of Hygiene and Bacteriology, University of Chicago.

The results obtained in this study, in general, confirm the observa-

tions made by previous investigators on the occurrence of carriers of the fixed types (I, II and III) of pneumococci; that in lobar pneumonia, the majority of instances of infection are due to organisms belonging to types I and II; that pneumococci of type III are responsible for the smallest percentage of cases of lobar pneumonia; that healthy persons intimately associated with cases of lobar pneumonia may harbor in their mouth secretions pneumococci of the same type as that isolated from the diseased individual; and that convalescents from lobar pneumonia may carry for a long time (one hundred and fifty days or more) the type of pneumococcus which was isolated during the infection.

The percentages of fixed type organisms seem to be approximately the same in the white and colored races within a given period.

The data show that the seasonal occurrence of fixed type pneumococci in the throats of normal individuals parallels the case rate of lobar pneumonia in Cook County Hospital. In other words, there are a greater number of normal individuals showing fixed type organisms in their throats during the pneumonia season than at any other time of the year.

From the evidence presented, the susceptibility of the individual seems to be of greater importance in determining an attack of pneumonia than the mere transmission of a so-called virulent variety of pneumococcus.

Type III pneumococci associated with lobar pneumonia are of high electrophoretic potential and of high virulence for white mice and human beings. On the other hand, the same type of organisms (as determined by serological methods) isolated from normal throats are of low potential and, in a parallel manner, of low virulence for white mice, and, as far as we can ascertain, seem to have no harmful effects on the human host.

The data indicate that electrophoretic potential measurements are more valuable as indices of virulence of various strains of pneumococci than virulence tests with white mice.

10. The Bacillus of Swine Erysipelas Causing a Distinctive and Severe Form of Erysipeloid Among Fish Handlers. MALCOLM J. HARKINS, Research Institute of Cutaneous Medicine, Philadelphia, Pa.

The somewhat similar lesions of erysipeloid of fish handlers and those of swine erysipelas in man and swine suggested a common bacterial

cause. Skin and subcutaneous tissue excised from infected areas revealed an organism having the morphological, biological and serological characteristics of the bacillus of swine erysipelas. Virulence of organisms and serological reactions in infected individuals were determined.

11. Studies of the Etiology of the Common Cold. W. C. NOBLE, JR., E. A. FISHER AND D. H. BRAINARD, Bacteriological Research Laboratory, Metropolitan Life Insurance Company.

I. A comparison of the flora of the upper respiratory tract in individuals in health, and with colds. In January, 1926, an experimental study was begun of the etiology of the common cold. Cases of acute coryza were selected for study from among the employees of the company who were suffering from the infection, and for comparison, a simultaneous study was made upon a number of healthy volunteer subjects.

In the work herein reported the clinical history was first taken, and then a thorough examination made of the nose and throat. Separate cultures were then taken with straight swabs from (1) the nose, (2) the tonsil, (3) the oropharynx. Cultures from the nose gave for the most part pure, or almost pure cultures of staphylococci, or diphtheroids. In the tonsillar cultures, green and hemolytic streptococci occurred in relatively high incidence. The oropharynx yielded a greater variety of types than the other regions investigated. Without exception, all our cases of acute coryza had symptoms of oro- and naso-pharyngitis. It was therefore decided to discontinue nasal and tonsillar cultures, and to make our study upon a single culture from each subject taken from the naso-pharynx with a bent swab.

Cultures taken in the winter and early spring from 21 healthy individuals showed the green streptococci to be the predominating organisms (49 per cent of the entire aerobic flora). Pneumococci comprised 3 per cent, staphylococci 18 per cent, indifferent streptococci 6 per cent, and hemoglobinophilic bacilli 1 per cent. In cultures from 11 acute coryza cases the green streptococci constituted 41 per cent, pneumococci 12 per cent, staphylococci 3 per cent, indifferent streptococci 9 per cent, and hemoglobinophilic bacilli 14 per cent. In acute coryza therefore, the basic normal flora as represented by the green streptococci and the staphylococci appears to be decreased relative to the other aerobic types, while the pneumococci, the indifferent streptococci, and the hemoglobinophilic bacilli are relatively increased. The case incidence likewise for the pneumococci and the hemoglobinophilic bacilli was increased in colds. These changes are also brought out in the serial culture studies.

In this preliminary study, no attempt was made to type serologically any of the organisms encountered.

II. The naso-pharyngeal flora of individuals in health and with colds, studied by the serial culture method. The work herein reported was commenced in February 1926. In March it was suspended to enable us to make a bacteriological study of so-called epidemic grippe. With the exception of this interruption, the study has so far been carried on for ten months.

Our problem has been to study the changes occurring in the naso-pharyngeal flora of healthy individuals when colds develop.

Cultures have been taken at approximately weekly intervals from 8 volunteer subjects, while they have been free from colds, and at more frequent intervals when colds have been contracted.

Our results may be summarized as follows: (1) There is a definite increase in the numbers of organisms when colds develop (7600, average for healthy subjects, to 16,000 average in colds). (2) The predominating basic normal types are represented by the Gram-positive cocci especially the green streptococci, hemoglobinophilic bacilli; Gram-negative cocci, and diphtherioids constitute other organisms encountered in smaller percentages in the flora of healthy subjects. (3) There is a relative decrease of the green streptococci as colds develop. There is an increase of the indifferent streptococci, the hemoglobinophilic bacilli, and the Gram-negative cocci. (4) In certain individual colds, bacterial types new to the subject appear, especially bacilli of the hemoglobinophilic group. These new types may become for the time being, the dominant organisms. Many of them show a tendency to persist for some time after the cold has passed, but in that event their comparative incidence becomes lower.

III. Naso-pharyngeal flora of cases of so-called epidemic grippe. In February and March, 1926, there was an extensive outbreak of so-called epidemic grippe in New York City. In March a study was made of the naso-pharyngeal flora of 27 cases selected from patients reporting for treatment at the Company's Clinic for employees. The study was not begun until after the peak of the epidemic had passed, but the cases selected were clinically typical for the outbreak.

The results show: (1) that there was an increase in the total number of organisms (an average of 7800 per grippe case to 1700 per normal control subject); (2) a comparative incidence of organisms as follows: hemolytic streptococci, 1 per cent; green streptococci, 36 per cent; pneumococci, 12 per cent; staphylococci, 15 per cent; indifferent strep-

tococci, 10 per cent; hemoglobinophilic bacilli, 8 per cent; Gram-negative cocci, 10 per cent; miscellaneous organisms chiefly diphtheroids, 8 per cent; of the total aerobic flora; (3) the predominating organisms were the green streptococci in 13 cases, pneumococci in 5 cases, staphylococci in 4 cases, indifferent streptococci in 2 cases, hemoglobinophilic bacilli in 1 case, *M. catarrhalis* in 1 case. Meningococci were found in 2 cases. There was thus no one bacteriological picture characteristic for the outbreak. Park and Williams reported a case incidence in New York of from 80 per cent to 100 per cent for hemoglobinophilic bacilli for the pandemic of 1918, and Williams, Nevin and Gurley of 92 per cent for the secondary outbreak of 1919–1920. Our average figures for 35 normal control subjects show, for the hemoglobinophilic bacilli, a case incidence of 23 per cent; and for our 27 grippe cases, of 45 per cent. The outbreak of 1926 is therefore characterized by its relatively much lower incidence for the hemoglobinophilic group of bacilli.

12. A Study of the Biology of B. fusiformis. JOSEPHINE S. PRATT, Fifth Avenue Hospital, New York City.

Pure cultures of fusiform bacilli isolated from the throats of man and rabbits were tested for cultural and immunological reactions and for pathogenicity. They fall into two groups, based on the ability of some strains, the minority, to ferment sucrose. There is no apparent immunity relationship, the sera of immunized rabbits fixing complement only in the presence of the homologous antigen. Pathogenicity is slight and variable. Fusiform bacilli occur normally in the throats of monkeys and guinea pigs, but no strains from these sources were studied.

13. Failure of the Mouse Test to Demonstrate the Presence of Type-I Pneumococcus in Sputum. An Unusual Instance. RUTH GILBERT AND C. K. DAVENPORT, New York State Department of Health, Division of Laboratories and Research, Albany, N. Y.

The use of the mouse method for the determination of the types of pneumococcus present in sputum is so widely recommended that an instance in which this procedure failed to demonstrate the presence of type I in a specimen containing both types I and III seems worthy of note. A specimen was received from a case of lobar pneumonia, on the third day of illness, and Avery's culture medium and a mouse were inoculated. Tests with the culture in Avery's medium yielded evidence of the presence of type I pneumococci. Upon learning of these

results, the physician administered antipneumococcus serum and late in the afternoon of the fourth day the patient's temperature which had reached 105.6 began to subside and continued to do so until normal was reached at midnight of the following day. Recovery was rapid and apparently complete. The mouse that had been inoculated was found dead at the end of 48 hours. Agglutination and precipitation tests made with material from the peritoneal vacity reacted with type III pneumococcus serum only and cultures of type III pneumococci were isolated from the peritoneal fluid and heart's blood. In view of these results, another specimen was requested and a mouse inoculated with it gave similar results with the agglutination and precipitation tests, but colonies of both types I and III were isolated from the heart's blood and peritoneal fluid.

14. Studies on Standardization of Antigen for Kahn Test. R. L. KAHN AND NATHAN NAGLE, Michigan Department of Health, Lansing, Mich.

Various lots of powdered beef heart occasionally produce antigens of somewhat different titer and sensitiveness in the Kahn test. This is believed to be due to variations in the amount of lipoids extracted from the beef heart. Antigens may be corrected to a standard titer of 1 + 1.1 (1 cc. antigen + 1.1 cc. normal saline) and to the required sensitiveness by various methods which will be fully described elsewhere. Two of these methods are briefly presented.

If an antigen gives a titer of more than 1 + 1.1, it is diluted with 95 per cent alcohol containing 0.6 per cent cholesterin. To determine how much dilution is required, two 5 cc. amounts of cholesterinized antigen are diluted with 0.75 cc. (15 per cent) and 1.5 cc. (30 per cent) cholesterinized alcohol, respectively. These 2 diluted antigens are now employed in 1 + 1.1 cc. titers with a half dozen or more weakly positive and a few negative sera, using standard Kahn antigen (obtained at the Michigan Department of Health or on the market) as a control. The diluted antigen which gives results practically identical with the control antigen is taken as the new standard and the entire amount of antigen is accordingly corrected with cholesterinized alcohol. Only 2 antigens in a large series required dilutions with more than 30 per cent cholesterinized alcohol. These 2 antigens gave titers of 1 + 1.6 and 1 + 1.7, respectively, and they both required 45 per cent dilution with cholesterinized alcohol to bring them to standard requirements. This dilution method may also be employed with antigens giving titers close

to 1 + 1.1. Occasionally, as little as 10 per cent dilution with cholesterinized alcohol will bring such antigens to the desired sensitiveness.

If an antigen gives a titer of 1 + 0.7, 1 + 0.8 or 1 + 0.9, it is concentrated as follows: Two 5 cc. amounts of the alcoholic extract are evaporated to dryness by means of a revolving fan. The residues are redissolved in 5 cc. and 10 cc. amounts, respectively, of the cholesterinized antigen. The modified antigens are filtered and tested in 1 + 1.1 titers with weakly positive and negative sera using the standard antigen as a control similarly to that described above. The antigen which gives results practically the same as the control is taken as the new standard.

Beginners with the Kahn test should procure sufficient standard Kahn antigen to use as a control in standardizing their antigen.

15. Relation between Appearance of Precipitate and Potency of Serum in Kahn Reaction. R. L. KAHN, ELIZABETH McDERMOTT AND NATHAN NAGLE, Michigan Department of Health, Lansing, Mich.

Precipitation reactions are considered four plus in the "Routine" Kahn test when definite flocculi are suspended in clear fluid in each of the 3 tubes. In such reactions, the bulk of precipitate is approximately 4 times as great in the first tube which contains 0.05 cc. of antigen dilution as in the third tube which contains 0.0125 cc. of this dilution. Aside from the difference in bulk, precipitates are not always identical in appearance, being more flocculent in some cases than in others. An attempt was made to find whether there is a direct relation between the appearance of precipitates and the potency of sera. It was found that four plus sera of comparatively weak potency, having from 4 to 80 reacting units as determined by the quantitative Kahn reaction, frequently showed heavy flocculent precipitates, while sera of high potency containing up to 400 or more reacting units, in many cases showed less heavy flocculi. Certain sera showing heavy flocculent precipitates in the first 2 tubes and much finer flocculi in the third tube were also found to contain comparatively large numbers of reacting units. It is recommended therefore that Kahn reactions in which the flocculation in the routine test is not marked and which are likely to be read, let us say, +++, +++, +++ as well as those which one might read ++++, ++++, +, should not be averaged to final readings of three plus but instead, should be checked either with a regular quantitative test (standard antigen dilution, 0.01 cc.; serum dilutions,

0.15 cc.) or with a two-tube quantitative test using 1:5 and 1:10 dilutions of serum. A serum giving a positive reaction in one or both of these dilutions, has either 20 or 40 reacting units and should have a final four plus designation.

In the case of weakly positive sera, the first tube of the Kahn test shows little or no precipitation due to the inhibitory effect of an excessive amount of antigen, the second tube more precipitation depending on the potency of the serum, and the third tube, containing the least amount of antigen, the most marked precipitation.

In the quantitative reaction, it is occasionally observed that the undiluted serum shows a finer precipitate than the 1:5 and subsequent serum dilutions. In such instances, the undiluted serum reaction is read positive on the basis of the heavy precipitates shown by the diluted serum.

In reading precipitation tests, it is important to use clear, thin-walled glass tubes which are free from scratches and absolutely clean.

16. Anaphylaxis. Studies V. Allergic Responses of the Embryonic Chicken Heart. NOBLE P. SHERWOOD, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

Studies were made on passive sensitization and reverse anaphylaxis of two- or three-day-old chick embryos. Anti-sera were prepared from chickens and rabbits. This necessitated an investigation of the viability of chick embryos at different temperatures, the regularity of the cardiac rhythm and the effect of various kinds and amounts of normal blood sera upon the heart rate, rhythm and amplitude. Methods for the study of the embryo either attached to or detached from the yolk were worked out. Lewis' observations on the viability and the effect of temperature on rate and rhythm of the excised heart were confirmed. Locke-Lewis solution was used in the bath. If embryos were selected whose rhythm was regular and high titred immune serum from chickens added to the bath, they would frequently apparently become sensitive to non-toxic doses of antigen. This was manifested by pauses in diastole and a decrease in rate and apparently in amplitude. As a rule, where the embryo was attached to the yolk, this would develop in two and one-half minutes after the addition of the antigen. Normal rhythm could frequently be restored by washing several times. When the same dose of antigen was again added no effect was observed except that occasionally there was some slowing. When reversed anaphylaxis was tested for and obtained, the phenomenon would appear in about thirty

seconds. A second addition of the same amount of anti-serum, after several washings, gave no response. Negative results in a short series were obtained using anti-serum prepared from rabbits. Permeability phenomena apparently exercised considerable influence in both toxic and sensitization phenomena. While reactions of this type may occur under conditions such as described by Lewis, yet it is thought that the phenomenon occurring under proper conditions is an expresion of anaphylaxis.

17. Anaphylaxis. Studies VI. Passive Sensitization of Turtles and Guinea Pigs Using Immune Serum from Chickens and Rabbits. NOBLE P. SHERWOOD AND C. M. DOWNS, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

Twenty turtles were injected with high titred immune serum from chickens and 16 with immune serum from rabbits. They were all tested, after varying intervals of time, for passive sensitization, using the cardiac response as a criterion. Tracings of both auricular and ventricular behavior were obtained. The antigen was injected intracardially. Desensitization and toxic phenomena were tested for. A positive reaction is indicated by a decrease in amplitude, more or less slowing and an apparent rise in tone. This is similar to the response obtained by Downs in actively sensitized turtles. Of the turtles receiving high titred immune serum from chickens, 6 gave positive reactions as compared to 1 reaction that may be positive out of 16 receiving immune serum from rabbits. The latter needs further confirmation. Observations were made on the shortest and longest time intervals after injection that positive reactions were obtained. Reversed anaphylaxis was apparently demonstrated once.

Twenty-eight young virgin guinea pigs were injected with anti-serum from chickens and tested for passive sensitization by the Schultz-Dale reaction. All were negative. A short series tested by the clinical method were also negative. This latter is in accord with Doerr's results.

A study was made of the cardiac response for guinea pigs using the technique described for turtles. While some results were apparently positive the series of both tests and normals was too short to serve as a basis for final conclusions.

18. Anaphylaxis. Studies VII. Active Anaphylaxis in Turtles. C. M. DOWNS, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

A short series of turtles injected several times with mammalian serum into the body cavity were tested for sensitization from three to seven days after the last injection. After pithing, the heart was exposed and the auricle and ventricle attached to a heart lever and the record made on a smoked drum. After a normal tracing had been made the antigen, in doses of from 0.3 to 0.7 cc., was injected into the ventricle. If reaction took place the same amount of antigen was again injected to test for desensitization. If a second reaction took place, both reactions were considered as toxic reactions. Sixty per cent gave a reaction which was presumably anaphylactic since no change took place on the second injection of antigen. The positive reaction consisted of a decrease in amplitude of both auricle and ventricle but more marked in the auricle. There was also a rise of tone and slight slowing. The reactions were identical with those observed by Sherwood and Downs in passive anaphylaxis in turtles.

19. The Rôle of Homologous Immune Serum in Microbic Dissociation.
PHILIP HADLEY, University of Michigan, Ann Arbor, Michigan.

The outstanding characteristics of microbic dissociation are described and the S, O and R culture types resulting from this reaction are related to certain cyclostages embraced in the bacterial life-cycle. The most characteristic and frequently observed differences between the S and the R forms of culture are catalogued. The frequent correlation between S type culture, high virulence and non-phagocytability on the one hand, and, on the other, the correlation between R type culture, non-virulence and phagocytability, are emphasized. The inciting causes of microbic dissociation are enumerated and, among these, the stimulus of homologous immune serum is considered in detail. It is pointed out that the action of immune serum cannot commonly be regarded as germicidal, although it is sometimes bacteriolytic. Its chief effect on the culture is to produce a dissociation with the consequent production of the R culture type. It is shown that this has been amply demonstrated *in vitro* in the case of several important pathogenic species, such as *Bact. lepisepticum,* the diphtheria bacillus, the pneumococcus and the streptococcus. Certain evidence also suggests that this reaction occurs in the body. Since the fact of microbic dissociation *in vivo* would serve to transform a virulent but non-phagocytable organism into a non-virulent but phagocytable organism, it would appear that the function of the bacteriotropic antibodies may be interpreted as that of producing *microbic dissociation in vivo.* It is suggested

that this is in reality the mechanism of the "preparing," opsonic action. Since no inciting agent is known that can compare with homologous immune serum in dissociation-provoking power, and since it is clear that such serum is not usually germicidal in marked degree, it is suggested that, in the future, the goal of much therapeutic endeavor will be to combat infections in the body through the use of substances that may possess dissociation-furthering power even though they may not possess significant germicidal action.

20. Microbic Dissociation and the Mutation Myth. PHILIP HADLEY, University of Michigan, Ann Arbor, Michigan.

With reference to the systematic aspects of bacteriology it appears that bacteriologists as a rule have occupied themselves in attempting to perfect schemes of classifications rather than in studying the nature and cause of bacterial variation. When new or unusual forms of culture have arisen, they have usually been looked upon as contaminations, involution forms or as "mutants." There exists at present among bacteriologists a marked tendency to discover mutants. But the author holds that none of these new forms, even though they manifest some degree of permanence in sub-culture, justify the use of the term, mutation. The reason for this is that the so-called mutants merely represent partially stabilized cyclostages in the life-history of the species concerned. It is pointed out that both d'Herelle and Bordet have set up the bacteriophage as an agent endowed with the power of producing mutations from normal cultures, and that the former has expressed the view that all fixed bacterial mutations are produced in this manner. The conclusions cannot hold true, because it is shown that all the bacteriophage can accomplish in this direction is to produce a dissociation of the culture characterized by the formation of new culture types, or cyclostages. All of these new culture forms, however, can be produced, although more slowly, by the culture itself without the intervention of the foreign bacteriophage. The "normal" production of these new forms, may be accelerated by the use of certain "incitants" to dissociation. The author concludes that the bacteriophagic "mutants" of d'Herelle and of Bordet are therefore only partially stabilized stages in the cyclogeny of the species. Furthermore, that no unequivocal case of true mutation is at present known among the bacteria.

21. Microbic Dissociation in B. Subtilis. M. H. SOULE, Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan.

When pure strains of *B. subtilis* are cultured in fluid mediums at least 2 types of organisms can be isolated, the normal or "S" form which is equivalent to that first described by Ehrenberg and later by Cohn, Koch, Klein, Eisenberg, Flügge and Chester, and an "R" or resistant form which varies morphologically and culturally from the classical type and which has not been found adequately described in the literature. In broth the "R" strain produces non-motile filaments often attaining a length of 200μ which rapidly agglutinate with the formation of a flocculent precipitate. The surface colonies on agar are somewhat irregular and flat with cuneate surfaces which sparkle by oblique, transmitted light. Microscopically these colonies cannot be differentiated from anthrax. This form does not liquefy gelatine quite as rapidly as the normal type, the colonies on gelatine are composed of twisted filaments and characterized by edges that are curled under.

Biochemically the two strains have been found identical in their reactions.

Inoculation of large volumes of fluid medium with the "S" type results in the production of "R" forms in proportion to the quantity of medium and the interval of active growth. Quiescent cultures do not dissociate and spores fix their own correlated vegetative culture type. Normal forms may be produced from "R" strains by inoculating large volumes of fluid medium but the percentage of "S" forms obtained from "R" types is much smaller than the percentage of "R" forms produced from "S" strains under the same conditions. The "R" type thus seems to be the stable form. The "S" form is more strongly antigenic. Immune serum has a marked dissociating power on its homologous germ and a stabilizing action on the opposite form. By the incorporation of "S" or "R" immune sera in fluid medium "R" forms can be obtained from "S" forms and "S" from "R" respectively.

In addition to pure laboratory cultures, recently isolated strains from hay infusions, air, water and milk have been studied. Both the "R" and "S" forms have been obtained from the above sources. It would seem that the "R" form being the more stable type must have been isolated and described before. *B. cereus* as described by Chester may be the "R" form of *B. subtilis*.

22. Functional and Morphological Changes in the Life History of Dermacentroxenus Rickettsi Wolbach, the Causative Agent of Rocky Mountain Spotted Fever. R. R. Spencer, Hygienic Laboratory, Washington, D. C.

Recent studies upon the behaviour and staining characteristics of the causative agent of Rocky Mountain spotted fever, *Dermacentroxenus Rickettsi* Wolbach, in the tissues of ticks and mammalian blood suggest at least three developmental phases in the live cycle of this parasite. These phases are: (1) A dormant or resting stage in hibernating ticks. (2) A virulent, highly fatal, phase in nymphal and adult ticks following feeding. (3) The phase in the mammalian host.

23. The Evolution of B. radiobacter from Mixed Cultures of B. and Pseudomonas radicicola. THOMAS E. RICHMOND, Ohio State University, Columbus, Ohio.

We have been able to show by growing cultures of certain legume, nodule forming organisms in mixture (*Bac. radicicola* and *Ps. radicicola*) that a third type of organism develops which can be identified as *B. radiobacter.*

B. radiobacter is not nodule forming as are the parent cultures from which it is derived although it is often mistaken for them. *B. radiobacter* is a non-symbiotic nitrogen fixer, and can be derived experimentally from mixtures of the nodule forming symbiotic types.

By nitrogen fixation and nodule formation on navy bean roots I have been able to show a great variation in the nitrogen fixing power of different strains of the nodule organisms common to the navy bean plant.

24. The Occurrence of Bacteriophage and Variation in Streptococcus Colony Formation. L. O. DUTTON, Methodist Hospital, Memphis, Tenn.

In a previous report concerning the bacteriophagic activity manifest against the streptococci some data were presented that indicated that certain colony variations from the normal were indicative of "mixed" cultures in the sense of being mixed with phage. By suitably varying the cultural conditions it has been found that very many strains of streptococci manifest these colony variations. It is a radical assertion to say that all strains of the streptococci occur as mixed strains. Additional data are offered to substantiate this contention. More detailed study of suitably selected strains of streptococci gives evidence of definite transmissible lysis, although it is not usually maintainable through many passages. The colony variations in question have been successfully induced in a normal strain by the addition of filtrates of streptococcus cultures that were thought to contain bacteriophage. Similar colony

variations have been observed in strains of organisms other than streptococci, which were capable of spontaneous lysis and capable of instituting transmissible lysis. Similar colony variations have been experimentally produced in a normal strain of *B. typhosus* by a definitely known anti-typhoid phage. It has also been possible to show that attempts at lysis of a mixed strain other than streptococcus show peculiarities of the phenomenon similar to that observed in the studies on streptococci. In view of such evidence it seems necessary to admit other manifestations than transmissible lysis and plaque formation, as criteria of the presence of the bacteriophage.

25. The Rôle of the Bacteriophage in Streptococcus Infections. III. As a Factor in the Recovery from Infection. L. O. DUTTON, Methodist Hospital, Memphis, Tenn.

Seven cases are reviewed in which the bacteriophage was administered as a therapeutic agent. The result of 5 recoveries out of this series indicates that there are possiblities in this direction for the control of streptococcus septicemia.

A review of 5 cases of streptococcus septicemia in which no therapeutic agent was used, and in which spontaneous recovery occured, indicates that the bacteriophage is probably a factor in the recovery of these patients.

26. A Study of the Toxicogenic Properties of Some Streptococci. MARTIN FROBISHER, JR., AND J. HOWARD BROWN, Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore, Md.

A review of literature, correspondence and experimental data shows that strains of streptococci regarded as the etiological agent in scarlet fever form a heterogeneous group, differing widely among themselves culturally and immunologically. Additional doubt as to the true nature of the etiological agent of scarlet fever is raised by reports in the older as well as recent literature of organisms other than streptococci for which there seems to be good evidence of etiological relationship to scarlet fever.

The evidence on both sides seems conflicting but also more or less convincing. Acceptance of either view makes it difficult to explain a number of puzzling things which have been observed during the study of scarlet fever.

An hypothesis is suggested which not only appears to reconcile some

of the conflicting views but provides an answer to many questions which have arisen in connection with the problem.

It is suggested that any suitable organism, usually *Streptococcus pyogenes*, may be induced to form scarlet fever toxin under the influence of a certain hypothetical principle, virus or second factor. The streptococci or other organisms found in etiological relationship to scarlet fever may be supposed, according to this hypothesis, to play a non-specific part. The unknown second factor is regarded as the specific agent. Neither alone is capable of causing scarlet fever. It is conceivable, according to the idea, that when non-toxicogenic organisms are subjected to the influence of the second factor, they may acquire the power of producing toxin. An organism having the power of causing scarlet fever may previously have been capable of merely maintaining a foothold in the throat or at most causing a localized infection.

In an experimental investigation of this hypothesis it has been found possible to induce two or three strains of toxicogenic streptococci to acquire temporarily, the power of forming toxin of considerable strength and neutralizable with scarlet fever antitoxin.

The method consisted in cultivating the non-toxicogenic strains in sterile Berkefeld "V" filtrates of, or in mixed culture with the toxicogenic strain and then recovering them. The methods of control have obviated the possibility of impure culture and error due to any of the original toxic filtrate.

It is believed to be indicated that not every cell of a given culture of non-toxicogenic streptococcus will acquire the toxicogenic property. It also seems to be indicated that the factor concerned in the transfer of this property may have moderate resistance to heat.

27. The Influence of Diphtheria Toxin on the Growth of Certain Bacteria. J. M. Sherman, C. N. Stark and Pauline W. Stark, Cornell University, Ithaca, N. Y.

The influence of diphtheria toxin upon the growth of bacteria has been tested. The toxin was added to beef infusion broth so that the resulting medium contained 30 m.l.d. per cubic centimeter. A number of bacteria tested showed no detrimental effect of the toxin upon their growth, but an exception was found in the case of a culture carried in this laboratory under the name of *Bacillus cereus*. With *B. cereus*, a small but definite retardation of growth in the presence of toxin has been repeatedly observed.

While it cannot be definitely stated that the factor contained in the

diphtheria toxin preparation which is toxic to *B. cereus* is the same factor which is toxic to higher animals, certain observations support that view: (1) Heating entirely destroys the factor which is inhibitory, (2) the toxic factor for *B. cereus* is apparently inactivated when sufficient antitoxin is added to neutralize the diphtheria toxin.

These observations suggest interesting possibilities: If the toxin is in fact toxic to some bacteria, it may be possible for such organisms to respond by the production of a specific antitoxin. As yet we have no evidence supporting this view. Since it is known that toxins may be destroyed by proteolytic enzymes, it is conceivable that the growth of some bacteria in the presence of diphtheria toxin would result in the destruction of its toxicity without impairing its antigenic properties.

We are greatly indebted to Dr. F. M. Huntoon, Director of Medical Service, H. K. Mulford Company, for supplying us with standardized and unpreserved toxin and antitoxin.

28. Efficient Production of Agglutinins. CHARLES A. BEHRENS, Purdue University.

It was desirable to determine whether or not high titre production depended upon the amount of the antigen injected at one time, or upon the frequency of stimulation of the body cells of the animal by the inoculation of small amounts of foreign protein. Consequently special attention was directed to dosage, total amount injected, and the frequency of the inoculations of the agglutinogen necessary to produce a satisfactory agglutinin formation.

It was shown that small doses of an agglutinogen inoculated not too frequently over a short period of time produced agglutinins of a higher titre than larger amounts extended over the same or longer period of time.

Similar experiments were conducted using various precipitinogens. In the production of precipitins the advantage of small dosage over larger amounts was further conclusively demonstrated.

The development of haemolysins employing these methods proved to be unsatisfactory except in the case of sheep red blood cells.

29. Observations on the Soluble Antigens of Bacterium Enteritidis. SARA E. BRANHAM AND ELEANOR M. HUMPHREYS, Department of Hygiene and Bacteriology, University of Chicago.

Toxic, bacteria-free filtrates of cultures of *Bact. enteritidis* grown in a protein-free medium, when injected into rabbits, stimulated the produc-

tion of agglutinins, precipitins, complement-fixing antibodies, and possibly antitoxins.

When these filtrates were concentrated by evaporation *in vacuo*, the products so obtained were better antigens than emulsions of *Bact. enteritidis*.

Protein could not be demonstrated even in the dry residue obtained when such a filtrate was evaporated to dryness. When this dry residue was dialyzed an opalescent fluid and a gray precipitate were obtained. Protein could not be demonstrated in either of these fractions. The precipitate contained carbohydrate. Further concentration of the opalescent fluid dialysate gave a liquid that showed very faintly positive reactions for tryptophane and histidine, and a very faintly positive ninhydrin reaction. Protein must therefore have been present. The vanillin and diazo reactions will detect tryptophane and histidine, respectively, in dilutions of 1:1,000,000. Calculated on the basis of these tests amounts of filtrate containing as little as 0.000,003 to 0.000,04 gram of protein led to definite antibody production. Whether or not so small an amount of protein can be held exclusively responsible for this antibody production, the possibility that it may do so should be borne in mind in interpreting results obtained with apparently protein-free materials.

30. Synthetic Media for Differentiation of the Typhoid-Paratyphoid Group of Bacteria. LUTHER THOMPSON, Mayo Clinic, Rochester, Minn.

Synthetic media consisting of various amino acids and inorganic sources of nitrogen may be used in identifying typhoid and paratyphoid organisms. In this work 28 typhoid, 10 paratyphoid A, and 11 paratyphoid B strains were used. With valine as a source of nitrogen all strains of paratyphoid B grew. 10 per cent of paratyphoid A strains grew, and but 3 per cent of typhoid strains. If glutamic acid is used as a source of nitrogen the percentages of positive growth are as follows: paratyphoid B 100 per cent, paratyphoid A 80 per cent, typhoid 18 per cent.

Synthetic media could be used to advantage as an additional cultural test, especially in cases of atypical agglutination among the paratyphoids.

31. Bacteria in the Supposedly Sterile Meconium. SEVERANCE BURRAGE, University of Colorado, Denver, Colorado.

Examination of nearly 100 meconium samples obtained from infants

just born and before suckling, showed the presence of bacteria in about 38 per cent of the samples. In the positive cases—and none was called positive unless the organisms were numerous—*Bacterium coli* was found in pure culture in about 50 per cent. The other positive cases usually contained a *Staphylococcus* in pure culture, but there were a few mixed cultures containing 2 or 3 species.

The babies from which the material was obtained were all born of healthy mothers, and with normal delivery.

The paper describes the methods of obtaining material; the care used to eliminate all chances of outside contamination; and the methods of making the cultures.

32. Antivenin Specificity. AFRANIO DO AMARAL, Antivenin Institute of America, Glenolden, Pa.

Specificity of antitoxins or antibacterial sera in regard to their respective antigenic principles or germs seems to be well established.

To the majority of immunologists this seems also to be the case in regard to the antivenomous sera or antivenins A few serum therapeutists, however, even among the most modern ones, apparently do not concur with those views. The Pasteur Institute of Paris, for instance, still states that its antivenomous serum, which is known to be specific for the cobra, is effective against various venoms, such as that of the rattlesnakes, the scorpions and the tarantulas.

This statement induced me to investigate the question in a general way by using several types of venom in cross experiments with a corresponding number of antivenins. The venoms used were from: *Crotalus terrificus*, *C. atrox*, and *C. adamanteus*, *Bothrops jararaca*, *B. jararacussu*, and *B. alternata*, *Micrurus corallinus* and *M. frontalis* (snakes) and *Tityus bahiensis* (scorpion).

The conclusions to be drawn from these experiments are the following: (1) Specificity is a general phenomenon among antitoxins. (2) "Group specificity" may also be found that will eventually justify the administration of a particular antivenin against the venom of species congeneric or closely related to that against which the antivenin is specific. (3) Application of anti-neurotoxic or anti-hemolytic sera or antivenins, however, against all neurotoxic or hemolytic venoms, no matter how zoölogically different the species from which the latter proceed may be, appears to be untenable.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

JOINT MEETING WITH SECTION O OF THE A. A. A. S.

SYMPOSIUM ON "SOME PROBLEMS IN SOIL BACTERIOLOGY"

1. The Fermentation Characters of the Legume Nodule Bacteria. I. L. BALDWIN AND E. B. FRED, University of Wisconsin, Madison, Wisc.

The fermentation characters of 60 strains of legume nodule bacteria, including members of the alfalfa, clover, pea, bean, soy bean, cow pea and dalea groups, and *B. radiobacter* have been determined. Agar media of low buffer capacity and carrying brom-thymol-blue as a hydrogen-ion indicator were used.

The fermentation characters of these organisms are sufficiently definite and pronounced to enable the separation of the organisms into groups corresponding to the cross-inoculation groups. An exception to this statement is found in the failure to separate the soy bean and cow pea groups.

By means of their fermentation characters the legume nodule bacteria may be divided into two broad groups (*a*) the acid producers which includes the alfalfa, clover, pea, bean and dalea bacteria. (*b*) Those which lower the hydrogen-ion concentration of the culture media, including the soy bean and cow pea bacteria. This separation corresponds to the separation made on the basis of flagellation. The first group are peritrichous while the second group have polar flagella.

In many of the cross-inoculation groups, subdivisions have been established. Within the alfalfa and clover groups, subdivisions on the basis of their fermentation characters are definite and clear cut. These groups correspond with the groups which have been established by other workers by means of cultural and serological tests. Considerable variation was exhibited among the members of each of the other cross-inoculation groups in their fermentation characters. In these groups, however, the differences between strains were less constant and gave less opportunity for classification into groups.

B. radiobacter may be distinguished from any of the legume nodule bacteria by its strong acid fermentation of dextrin.

Comparison of the reactions secured on nitrate, yeast water and nitrogen-free media shows that these organisms produce larger amounts of acid on nitrogenous media. This is probably merely a reflection of a more vigorous growth.

Many of the cultures apparently withdraw acidic radicals from the medium much more rapidly than they do basic radicals. A comparison of the reactions secured on the nitrate and non-nitrogenous media indicates that the nitrate radical, in particular, is rapidly withdrawn from the culture media by the organisms. However, the fact that many of the cultures produce an alkaline reaction in non-nitrogenous media indicates that some other acidic radical than nitrate is also heavily drawn upon. Evidence from other investigations would indicate that the phosphate radical is used in considerable amounts.

2. *The Taxonomy of the Legume Bacteria.* R. E. BUCHANAN, Iowa State College, Ames, Iowa.

(No abstract.)

3. *The Course of Nitrate Accumulation in Soils Following the Growth of Crops.* T. L. LYON, Department of Agronomy, Cornell University, Ithaca, N. Y.

Determinations of nitrate nitrogen were made in samples of soils on which various crop plants had grown the previous year. Nitrate nitrogen was also leached at intervals from soils contained in large cans in which plants had previously grown. Characteristic differences in the quantity and rate of nitrate nitrogen accumulation are noted.

In years succeeding the growth of the crops whose effect on nitrate accumulation has been observed, a test crop was grown. The yields of the test crop are compared with reference to the quantity and rate of nitrate nitrogen accumulation. These comparisons indicate that the rate as well as the quantity of nitrate nitrogen accumulation influences the yield of the succeeding crop. This is because a succeeding crop with a short growing season will be favorably affected by a preceding crop that produces a rapid accumulation of nitrogen, while a succeeding crop with a long growing season may receive more benefit from a slower accumulation of nitrogen. For instance barley yielded relatively better than maize following vetch, and maize relatively better than barley following oats. The rate of nitrate accumulation was rapid following maize and slow following oats.

4. *The Nature of Soil Organic Matter and the Rôle of Microörganisms in its Formation and Decomposition.* SELMAN A. WAKSMAN, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

The problem of soil organic matter is one of the most complicated

problems in soil science and has had centered upon it in the past the attention of the soil physicist, the soil chemist and the soil geneticist as well as of the agronomist. The contributions of the bacteriologist were limited largely to the decomposition of certain constituents of the organic matter or to measuring the formation of one of the intermediary products or final products formed from the decomposition of complex organic substances. In reviewing the work carried out at the New Jersey Agricultural Experiment Station on the decomposition of complex organic substances by microörganisms, the author calls attention to the fact that soil "humus" is derived from two different sources: (1) Certain constituents of the original plant materials added to the soil which resist decomposition by microörganisms, namely, the lignins, and, to a more limited extent, the waxes, tannins, etc. (2) Substances synthesized by microörganisms, in the process of decomposition of 70 to 80 per cent of the constituents (celluloses, pentosans, etc.) of the natural organic materials added to the soil. The author reviews in detail the various processes of decomposition of the celluloses, pentosans, proteins and other constituents of plant materials as carried out by various groups of microörganisms finally leading to a synthesis of organic matter. The synthesized materials undergo decomposition in their turn, leaving certain constituents of the cells of microörganisms which are resistant to decomposition. Soil humus is thus found to be very complex in nature and should in no way be compared with the dark colored substances obtained by treatment of organic materials with strong acids or with certain complexes isolated from the soil by the use of chemical reagents. Very little is known of the decomposition of this humus in the soil by microörganisms. The results tend to indicate that the actinomyces and certain non-spore-forming bacteria play a very important part in the process.

5. *Studies on the General Soil Flora.* H. J. CONN, Agricultural Experiment Station, Geneva, N. Y.

A study of the soil micro-flora is very complicated on account of the great variety of microörganisms that may be present and the large number of different conditions that may influence their growth. Formerly the problem was attacked by roughly quantitative methods on the one hand, and so-called "qualitative" methods on the other, the latter dealing almost wholly with the bacteria taking part in the nitrogen transformations. Yet it has all the time been quite generally realized that quantitative methods are almost meaningless, and that bacteria of other

kinds than those concerned in nitrogen changes are really the more numerous in soil although less understood.

The first methods suggested for studying the general soil flora came from Hiltner and Störmer in Germany and from Chester in the United States. The author began work along these lines in 1909 and has been gradually adding new methods of attack. Progress was at first discouragingly slow. The first real contribution of importance was the development in 1917 of a method for staining bacteria in soil infusions, although its value in connection with soil flora studies was not realized at the time. The second important contribution was the development of methods for studying and classifying the non-spore-forming bacteria of soil, the classification depending largely upon the changes in morphology which they pass through in laboratory culture. The possibility of classifying these little understood organisms is pointing out methods of attack for their further study.

Most recently Winogradsky in France has shown a new method of applying the author's direct microscopic method to the study of the general soil flora. He has pointed out that it can be used to study the types of organism that develop in soil when any factor is introduced to change the normal soil conditions. Winogradsky's method is without question the most valuable contribution yet made to the subject.

The important problems at the present time are: first to learn the function of the little understood non-spore-formers in soil; and secondly to learn how different soils compare when studied by the technic of Winogradsky. Both these lines of investigation are being pursued in the author's laboratory.

6. *Influence of Environmental Factors on the Activities of Aerobic Bacteria Concerned in the Decomposition of Cellulose in Soils.* René J. Dubos, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Very active cellulose decomposing bacteria were isolated from soils by using the following medium; $NaNO_3$, 0.50 gram, K_2HPO_4, 1.0 gram, $MgSO_4$, 0.50 gram, KCl, 0.50 gram, $FeSO_4$, 0.01 gram, distilled water, 1000 grams; 5 cc. portions of this medium are introduced into test tubes containing a strip of filter paper which is partly immersed in the medium.

The slightly alkaline reaction of the medium (pH 7.5) favors the growth of bacteria while it retards the growth of fungi. The low concentration of nitrogen shortens the incubation period of bacteria, since growth and cellulose decomposition can be recorded after thirty-six to

ninety hours at 28°C. Growth is obtained even when only one or a very few cells are used for inoculation; this renders possible the use of the dilution method for determining the numbers of cellulose bacteria.

The influence of environmental factors on cellulose decomposition was studied by the following methods: (*a*) direct microscopic examination, (*b*) determination of the numbers of cellulose decomposing bacteria and fungi, (*c*) rate of CO_2 evolution, (*d*) amount of cellulose decomposed, when cellulose is added to normal soils, or to sterile soils inoculated with pure and mixed cultures.

The optimum conditions for the activities of cellulose bacteria seem to be: a temperature of 28°C., 50 to 70 per cent of the moisture holding capacity of the soil, a neutral or alkaline reaction, an abundant supply of nitrogen. The development of bacteria in some acid soils is accounted for by the fact that they become more tolerant toward acidity in the presence of a low concentration of nitrogen salts.

Under a set of conditions such as described above, cellulose decomposing bacteria probably play as large a part as fungi in the process of cellulose decomposition in the soil.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

7. *Use of Dyes in Media for the Identification of Soil Microörganisms. Preliminary Note on their Use in the Isolation of the Root-Nodule Bacteria of Legumes.* Harold W. Batchelor and Irvin H. Curie, Ohio Agricultural Experiment Station, Wooster, Ohio.

Twenty-nine dyes comprising 9 different chemical groups were used in a concentration of 1:40,000 in yeast-water mannitol agar. The plates were incubated at 28°C. for a maximum of two weeks for the rapid growing organisms and three weeks for the slow growing organisms. Long periods of incubation should be avoided if typical adsorption is to be obtained. A source of intense light such as used for dark field illumination is placed at an angle of about 35 degrees above the stage of a low power compound microscope to illuminate the colonies. No transmitted light is used.

Sub-surface colonies of the nodule bacteria, appear as unstained, white, opaque, lenticular forms with no evidence of a stained nucleus. Sub-surface colonies of the non-legume bacteria exhibit a degree of specificity in the adsorption of certain dyes. They are either chromogenic, completely stained, partially stained with apparent nucleus, or unstained "phantom" forms. The following dyes which have been used

successfully are listed in the order of their value: Poirrier's blue, alkali blue, nigrosin, orcein, methyl blue, pyronine, azure 1, and eosin. Poirrier's blue, alkali blue, nigrosin, or methyl blue when used in a concentration of 1:80,000 in media together with eosin at double that concentration give excellent plates.

A correlation of dye adsorption with the chemical constitution of the dye has been found in one case. A certain mucilagenous non-legume bacterium, as yet unidentified, adsorbs only dyes of the thiazin group. The dye is adsorbed by the gum and not by the organism.

The method has been used with success in the isolation of bacteria from peas, soybeans, cowpeas, alfalfa, and red clover which had been grown on Volusia Silt Loam.

8. *The Grouping of Different Strains of Pseudomonas Radicicola of Soja Max According to the Bacteriostatic Effect of the Pararosanilin Dyes.* WILLIAM H. WRIGHT AND ROBERT M. SIMINGTON, College of Agriculture, University of Wisconsin, Madison, Wis.

Detailed studies have been made of strains of *Ps. radicicola* isolated from soils from China, Manchuria, Japan, the Philippines, Florida, Louisiana, Mississippi, Alabama, Texas and Virginia. In the preliminary experiments 42 aniline dyes were studied. Only 3, all pararosanilins, were found of any value. The earlier work reported by the senior author in 1924 was confirmed for the biotypes.

Although all strains were *Gram-negative* there was great variation shown in their tolerance for the rosanilines. Hexamethyl pararosanilin gave the best results. The following conclusions are indicated:

(1) A concentration of hexamethyl pararosanilin (crystal violet) of 1:25,000 is necessary to stop growth of some strains (Type B) and as little as 1:150,000 will completely inhibit others (Type A). (2) The frequency distribution curves of the strain groupings according to inhibiting dye concentration indicate the types. (3) The differences in the strains are similar to Churchman's "strain within a strain" variants and can be shown with the Churchman divided plates.

9. *Certain Fermentation Products of the Root Nodule Bacteria of Legumes.* J. A. ANDERSON, E. B. FRED, AND W. H. PETERSON, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis.

Xylose, glucose, sucrose, lactose, and mannitol were fermented by two strains of root nodule bacteria from alfalfa. The fermentations,

which proceeded very slowly, resulted in the formation of small amounts of acids. The acids obtained from cultures of the different carbohydrates were apparently the same in all cases; namely, lactic, acetic, and pyruvic acids, a condensation product of pyruvic acid, and very small amounts of butyric acid. Quantitative data were obtained concerning the composition and amounts of the various acids.

The finding of pyruvic, lactic, and acetic acids is of considerable interest in connection with the latest conceptions of alcoholic and lactic fermentations, as well as of carbohydrate metabolism in muscle tissue.

10. Cross Inoculation Studies with Cow Pea and Soy Bean Nodule Bacteria. O. H. SEARS AND W. R. CARROLL, Illinois Agr. Exp. Sta., Urbana, Ill.

Previous observations that cow pea (*Vigna sinensis*) and soy bean (*Soja max*) nodule bacteria may cross inoculate under certain conditions have been confirmed.

Twenty pure cultures of organisms isolated from soy bean nodules, in this and in other laboratories, produced abundant nodule formation upon cow pea and soy bean plants. In no case did soy bean nodule bacteria fail to produce nodules upon seven different varieties of cow peas and soy beans respectively. Nine cultures of cow pea nodule organisms produced nodules in abundance upon the cow pea plant but failed to infect the soy bean. Eight other pure cultures isolated from cow pea nodules infected both cow pea and soy bean plants. The cow pea cultures which failed to inoculate soy bean plants are serologically homologous and do not agglutinate any of the other strains studied.

11. The Viability of Rhizobium Legumenosum Frank and Rhizobium Radicicolum Beijerinck. DAN H. JONES, Ontario Agricultural College, Guelph, Canada.

Stock cultures of *Rhizobium* species grown on modified Ashby's agar in small Freudenreich flasks were kept at room temperature in the laboratory from May, 1915, to May, 1926. The cotton plugs of the flasks had been sealed with wax to prevent evaporation. After eleven years' storage these cultures were tested for viability by plating out small quantities on modified Ashby's agar minus calcium carbonate. All plate cultures gave typical *Rhizobium* colonies. The cultures used in the test had been originally isolated from red clover, white clover, crimson clover, sanfoin, alfalfa, sweet clover, field pea, sweet pea, hairy vetch, field bean, soy bean and cow pea.

Plate colony counts were made to determine the number of living organisms in cultures of *Rhizobium radicicola* Beijerinck (alfalfa strain), grown on modified Ashby's agar slopes and kept for varying lengths of time in two ounce Blake square bottles which had been tightly corked after growth had developed.

Cultures 2 months old gave an average count of	5,000,000,000
Cultures 12 months old gave an average count of	1,400,000,000
Cultures 15 years old gave an average count of	110,000,000

The 15-year-old cultures had been stored in the attic where the temperature ranged from over 100°F. at times in summer to −20°F. at times in winter.

12. A Bacteriologic Study of the Spanish Green Olive. HENRY FIELD SMYTH, School of Hygiene and Public Health, University of Pennsylvania, Philadelphia, Pa.

This paper reviews the work of three years spent partly in this country and partly in Spain investigating spoilage factors in the curing, packing and marketing of Spanish green olives.

Green olives are treated with caustic to soften the skin and remove bitter principles, washed in water and then placed in 10 per cent brine in casks where they undergo a natural gassy lactic acid fermentation, primarily bacterial but later possibly aided and completed by yeast action. Originally alkaline, the brine becomes gradually acid, increasing normally by a definite curve to a final lactic acidity around 1.25 per cent. The keeping quality of green olives depends on: (1) degree of caustic removal in washing, (2) steadiness of rise of acidity dependent on clean methods and proper temperature, (3) preservative action of brine, (4) exclusion of air in packing.

Failure at any one point may result in spoilage during curing or after shipment or packing.

The maintenance of a proper degree of acidity is the one most important factor and the addition of lactic acid to olives of low acid value will prevent spoilage development.

Characteristic spoilage, termed zapatera, is detectable by taste, odor and consistency, and is always associated with lowered acid values. While never endangering life or even health, this spoilage may result in losses of thousands of dollars to the packers.

Spoilage is not due to any one organism but to one or more of a group of spore forming, proteolytic, facultative rods normally present in the

soil of Andalusia. These organisms develop well in brines up to and well over the salt content of olive brine, but are inhibited by lactic acid.

Determination of pH values by means of brom-phenol blue is the best single check on the keeping quality of green olives. They should test to pH 3.8 or, better, pH 3.6 or 3.7.

Type B *Cl. botulinum* has been isolated from orchard soil but never Type A, and there are no records of botulinus poisoning from green olives. This is due to the absence of Type A and the combined action of salt and acid inhibiting the growth of Type B.

13. The Value of Lactic Acid in the Preservation of Mayonnaise Dressing and Other Products. MIRIAM S. ISZARD, School of Hygiene and Public Health, University of Pennsylvania, Philadelphia, Pa.

Mayonnaise dressing and similar products are foodstuffs subject to rapid deterioration due to their high organic content. These products are difficult to preserve because of their complex and easily alterable composition.

Experimentation has shown that the type of spoilage is a process of fermentation due to bacterial action. In 50 spoiled samples examined, a Gram-positive, spore-forming bacillus was isolated. This organism was doubtless the causative organism of the spoilage, since when large quantities of this organism were inoculated into a freshly prepared product the same type of spoilage could be demonstrated in a week's time, whereas the normal product required anywhere from one to three months' time.

Analyses of all the ingredients entering into this product showed this same organism to be present in one of the ingredients. Because of the chemical nature of the ingredient and the fact that the organism was a resistant species, it was impossible to sterilize the ingredients before mixing.

Therefore, the only feasible solution of the problem was to increase the hydrogen ion concentration of the finished product to the point where bacterial development was inhibited. Pure lactic acid was used in this experimental work. Various amounts of lactic acid were added to the product and the product held to determine the preservative value of the acid. It was found that 1.75 per cent acidity (expressed as lactic acid) was the least amount necessary to prevent bacterial deterioration of the product over a period of five months.

Lactic acid treatment of such products seems to be a feasible method of arresting bacterial spoilage; first, because this acid is not harmful

from a health point of view, and secondly, because the amount necessary to use does not alter the taste as would vinegar were it employed in sufficient amounts to arrest bacterial development.

14. A New Fermentation Yielding Butyl and Isopropyl Alcohols. K. Morikawa and S. C. Prescott, Massachusetts Institute of Technology, Cambridge, Mass.

A new type of organism causing a fermentation process yielding butyl and isopropyl alcohols has been studied. This organism is not identical with previous species which have been described as butyl alcohol producers and the name *Bacillus technicus* N. sp. Prescott and Morikawa has been provisionally proposed. The biochemical characters of the organisms have been carefully studied, and it is shown to be a vigorous gas producer in the earlier stages of fermentation with rapid sugar consumption and comparatively quick increase in acidity. Later the changes are slower and appear to consist of the formation of butyl and isopropyl alcohols without the formation of acetone or ethyl alcohol. The yield of alcohols is better in fairly concentrated mashes, that is, those having 12 per cent or more of sugar as glucose, while in solutions with low concentration the yield is relatively small. The organism appears to be very sensitive to the presence of certain salts and to be stimulated by the addition of small amounts of calcium carbonate or dibasic ammonium phosphate. Various types of carbohydrates have been studied, and the highest yields have been obtained with rice which has undergone hydrolysis by the action of the enzymes produced by *Aspergillus oryzae.* It is believed that this fermentation may have industrial applications in the production of these solvents.

15. A Blood-red Yeast from Milk. (Exhibit.) Anna C. Brush, introduced by Jean Broadhurst, Teachers College, Columbia University, New York City.

Twice during the winter of 1925–6, bottled milk from a New York Company showed a blood-red surface growth. Repeated efforts to isolate *Serratia* failed, no color colonies being obtained with agar or any other culture medium. Finally yellow corn meal plates were used, and a slow but luxuriant blood-red growth was obtained, from which an oval budding yeast was isolated. This yeast is colorless on agar, gelatin, broth and all culture media except milk and corn meal, where the color is a deep red, very different from the salmon-pink, brick-red to bronze of other red yeasts. These results suggest that the frequent reporting of *Serratia* in milk may sometimes be due to this unusual yeast.

16. *The Preservative Action in Catsup of Salt, Sugar, Benzoate and Acid.* CARL S. PEDERSON AND ROBERT S. BREED, Agricultural Experiment Station, Geneva, N. Y.

An analysis of 16 brands of catsup showed a decided variation in the amounts of acid, sugar, and salt present. Each of these brands was inoculated with 32 bacteria and 2 yeast cultures, isolated from spoiled tomato products, to determine the relation between the amounts of acid, sugar, and salt present and the possibility of spoiling the product. The bacterial cultures were all Gram-positive, non-spore-forming rods and were of 5 quite different types. Four of the 5 groups produced acid and gas, and caused swells in tomato products, while the fifth group produced acid without gas, and slime in tomato products. Other morphological and fermentation characters are correlated with the characteristics mentioned.

It was found that 1.0 per cent of acetic acid, 5.0 per cent of salt, or 0.2 per cent of sodium benzoate was required to stop growth of all of the organisms used, but certain types were less resistant to these ingredients. Sugar was found to be very ineffective, 35 per cent inhibiting the growth of certain types only. Combinations of sugar and salt proved very effective, 15 per cent of sugar and 3.5 per cent of salt being sufficient to stop growth of all except one yeast. Combinations of sugar or salt with acid, on the other hand, did not lower appreciably the amount of acid required.

17. *The Relationship Between the Hydrogen Ion Concentration of Egg White and the Growth of Anaerobes.* C. N. STARK AND PAUL FRANCIS SHARP, Cornell University, Ithaca, N. Y.

It has been found that the hydrogen ion concentration of egg white changes rather rapidly from about pH 7.6 to pH 9.5, due to the loss of carbon dioxide, if the freshly laid egg is kept a short time in a well-ventilated place. This change in pH should be sufficient to affect markedly the growth of microörganisms in the egg white. Sharp and Whitaker have found that egg white, corresponding to the pH of the white of fresh eggs (about 7.6), permitted growth of a number of aerobes, while egg white corresponding to the pH of eggs which are in carbon dioxide equilibrium with normal air (pH about 9.5) was apparently germicidal. A somewhat similar study has been carried out with a number of anaerobes with the same result, that is the anaerobes were found to grow in egg white with a pH corresponding to that of a fresh egg, while no growth was found in egg white with a pH corresponding

to that of eggs in carbon dioxide equilibrium with normal air. These observations should be of practical importance in regard to the storage of eggs. They also offer an explanation for the contradictory results obtained by several invsstigators in studying the germicidal action of egg white, in that in some cases probably egg whites with a low pH, and in other cases egg whites with a high pH were used as experimental material.

18. The Quantitative and Qualitative Distribution of Lactobacilli in Milk.
J. M. SHERMAN AND C. N. STARK, Cornell University, Ithaca, New York.

The approximate quantitative occurrence of lactobacilli in milk has been determined by inoculating sterilized skim milk with dilutions of market milks, incubating at 37°C. for two weeks, and then determining the amount of lactic acid produced. Over 1.1 per cent acidity, as lactic acid, was considered as positive for the presence of lactobacilli. The integrity of this conclusion was checked many times by microscopic examination. In all cases in which a high acidity was obtained, large numbers of characteristic Gram-positive rods were observed. Tests have been made upon 233 samples of milk of which 66 samples were of grade A quality (New York City classification), while 167 samples were ordinary milks of indifferent quality. A summary of the results obtained follows:

	GRADE A MILK	ORDINARY MILK
	per cent	*per cent*
<1 per cc.	29	6
>1 per cc.	37	30
>10 per cc.	22	38
>100 per cc.	9	20
>1,000 per cc.	3	4
>10,000 per cc.		2

Since lactobacilli of the *bulgaricus* and *acidophilus* types will grow at 45°C. while the *casei* types will not, a convenient method of differentiating these groups was available. Also, the *casei* types will grow at 15°C. while the *bulgaricus* and *acidophilus* types cannot. By the use of these methods it has been found that *Lactobacillus casei* is generally present in milk in considerably greater numbers than are either *Lactobacillus bulgaricus* or *Lactobacillus acidophilus*. In the Grade A milks examined

only 12 per cent contained *bulgaricus* or *acidophilus* in numbers as great as 1 per cubic centimeter, while 71 per cent contained *casei* types in numbers as great as 1 per cubic centimeter. With ordinary milk, only 2.3 per cent contained *Lactobacillus bulgaricus* or *Lactobacillus acidophilus* in numbers exceeding 10 per cubic centimeter while more than 10 per cubic centimeter of *Lactobacillus casei* were found in 94 per cent of the samples.

19. The Types of Hemolytic Streptococci in Certified Milk. W. D. FROST, MILDRED GUMM AND ROBERT C. THOMAS, University of Wisconsin, Madison, Wis.

In a former paper by Brown, Frost and Shaw, the results obtained by the detailed examination of the hemolytic streptococci in certified milk, for a period of two months, was reported.

The present paper considers the results obtained, by following the methods suggested in the previous paper, in a study of the same milks, extending over a period of a year.

Composite samples from groups of about 10 cows were used. They were collected about once a month. One thousand and one group samples were examined involving the milk from about 1000 cows. Six-hundred and twenty-three samples, 62 per cent, showed hemolytic colonies of the beta type. Of these only 292, or 29 per cent of the total number of samples yielded cultures which hemolyzed in the test-tube or true beta type hemolytic streptococci.

The number of hemolytic streptococci in the samples was usually small. In 197 of the 292 samples, or about 67 per cent, the number was less than 1000 per cubic centimeter. In 272 of the 292 samples, 93 per cent, the number was less than 10,000 per cubic centimeter.

A detailed study of these streptococci showed them to belong to 8 different types or species. The *Streptococcus epidemicus* Davis was found in 2 cows. These cows had either a low grade of infection or were merely carriers as there were no pronounced clinical symptoms or physical change in the milk in either case.

20. The Use of the Blood Agar Plate in a Large Certified Milk Dairy. MILTON E. PARKER, Walker-Gordon Company, Plainsboro, New Jersey.

The sanitary control of certified milk production by means of the blood agar plate has been practiced with considerable success in a large certified dairy for over a year.

An analysis has been made of the individual records of 749 cows whose milk was examined by means of the blood agar plate according to the methods described. Of this number, 337 (native) animals represent the progeny of proved sires while the remaining 412 (alien) cows have been collected from different accredited farms in the country. Seventy per cent of the alien animals and 73 per cent of the natives thus examined had streptococcic infections including 36 per cent of the alien cows and 22 per cent of the natives with hemolytic streptococcus infections. The herd averages of these two groups indicated that 71 per cent of the animals had streptococcic infections including 30 per cent with hemolytic streptococcus in their milk.

From a study made of the records of the native cows, it appears that with increasing age the frequency of streptococcic infection tends to increase particularly after the fifth lactation period.

It has been a laboratory practice to exclude from production any cows producing milk which gives a blood agar plate count, under the conditions described, in excess of 10,000 colonies per cubic centimeter. The results of the analysis of 2070 blood agar examinations made upon the milk of 749 cows seems to indicate that such a figure is a fair one.

The blood agar plate affords certified milk production protection from the invasion of *Streptococcus epidemicus* and the inclusion within the herd of cows producing milk of high bacterial count.

21. *Some Aspects of the Methylene Blue Reduction Test of Milk.* H. R. THORNTON AND E. G. HASTINGS, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisc.

Methylene blue is an indicator of oxidation-reduction intensity. In milk the reduction intensity is dependent mainly upon the dissolved oxygen. Bacteria consume oxygen. Thus this test is a measure of bacterial activity.

A concentration of 1 part dye to 200,000 parts milk gives approximately the same results as a dye concentration of 1:100,000. Therefore extreme accuracy in measuring the sample of milk is not necessary. A 10 cc. dipper is sufficiently accurate for this purpose. In routine work, sterility of the dipper or of the dye solution is not necessary if the reduction times are not read after five and one-half hours. In its present form this test should not be considered reasonably accurate after the five and one-half hour period.

The two great inaccuracies in this test are: different rates of oxygen consumption by bacteria and sweeping of bacteria out of the milk by the rising butterfat.

There is only a general correlation between this test, the standard plate count, and the keeping quality of the milk. There is no reason to consider the reduction test more inaccurate than any of the other methods of estimating the bacterial condition of milk. Indeed, we believe that milks may be divided into large groups as accurately by this test as by any other. The methylene blue reduction test has the distinct advantage of being inexpensive and simple.

22. How Accurate is the Quantitative Plate Count? WILLIAM H. WRIGHT AND H. R. THORNTON, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisc.

The data here presented are the result of 2330 counts of milk plates poured in duplicate series, 100 plates to a series. Extreme care in technic was attempted and as many variables eliminated as was consistent with practicability. For example, 10 cc. of milk were diluted in 990 cc. of physiological salt solution instead of 1 cc. in 99 cc.; 10 cc. of agar were used in Petri dishes of uniform size and the plates were incubated in piles only three deep. A variation of less than 100 per cent within a series cannot be depended upon. The extreme variation in these experiments was 1500 per cent. The average coefficient of variability for all counts made was 25 per cent. The high and low extremes were 57 per cent and 15 per cent. A variation of less than 100 per cent between two dilutions of the same milk cannot be depended upon. The extreme variation between the means of two dilutions in these experiments was 400 per cent. The counts of two workers gave the same coefficient of variability while the total count of one worker was 10 per cent higher than that of the other. This was apparently due to the difference in eyesight of the workers. The results of these biometric analyses indicate that this method is no more accurate for grading milk than other methods available.

23. The Fixation of the Bacteria in Market Milk Samples. WM. E. COLE, PAUL W. ALLEN AND GEO M. CAMERON, University of Tennessee, Knoxville, Tenn.

Because of the need of market milk control by towns, villages and communities which are unable to support a bacteriological laboratory, a study of the problem of fixing the bacteria of market milk samples has been undertaken. A fixative has been sought which would fix the bacteria so that they could neither multiply nor disintegrate thus making it possible to make direct bacteriological counts of milk samples several

days after they have been taken. If a method of this kind could be worked out it would then be possible for small centers of population incapable of maintaining a bacteriological laboratory to send samples to a central service laboratory at some distance for bacteriological analysis.

Some of the materials experimented with as fixatives are: listerine, lysol, phenol, potassium permanganate, formaldehyde, mercurochrome, mercuric chloride.

Results on several hundred samples to date have shown that one part milk to one part listerine fixes the bacteria so that several days later the counts are the same as the original counts.

24. Sources of Thermophilic Contamination in the Canning of Peas and Corn. E. J. CAMERON, C. C. WILLIAMS, AND R. J. THOMPSON, National Canners Association, Washington, D. C.

During the canning season of 1926 field work was conducted in an attempt to define major sources of infection by spoilage organisms.

During the pea pack it was found that wooden brine vats were capable of becoming serious sources of infection, resulting from overnight accumulation of thermophilic spores. Later work, in connection with which numerous wooden tanks were sampled, showed that some of these contributed very little contamination but were nevertheless considered of potential danger. In two instances where contamination was excessive, lining these vats with tin plate served to keep numbers of spores within safe limits. The blanching and filling systems were also involved in adding to contamination by these organisms.

In the canning of corn thermophilic infection appears to result, not only from the use of wooden brine vats, but also from neglect to properly clean the cooking and filling system.

Tests for spoilage thermophiles on peas entering the canning plant were uniformly negative. Their presence on raw cut corn was suspected on rare occasions.

Tests made upon cane sugar showed that this substance contained definite numbers of thermophilic spores, and while the resultant direct contamination from this source was not extreme, considerable significance was attached to the fact that sugar afforded a constant means of entrance to the canning system.

In general, it may be stated that thermophilic contamination in the canning of peas and corn, to the extent necessary to indicate danger from under-sterilization, appears to be entirely a product of the canning plant and represents undue accumulation in those portions of the can-

ning system subjected to heat. Sugar is the only definite external source as yet established to account for their origin in the canning plant.

25. *The Importance of Some Heterotrophic Microorganisms in the Solution and Precipitation of Iron.* R. L. Starkey and H. O. Halvorson, Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn.

Equations have been developed which indicate that the concentrations of hydrogen-ions and oxygen are factors of primary importance in determining whether oxidation or reduction of iron will occur in systems where an excess of ferric hydrate exists. Experimental results are interpreted in the light of these equations.

Under aerobic conditions microörganisms may effect solution of iron as a result of acid formation; under anaerobic conditions microörganisms may dissolve and reduce iron present as ferric hydrate as a result of decreasing oxygen pressure and formation of acid and this may occur at reactions close to neutrality. Organic compounds of iron may form subsequent to solution of iron by microörganisms in organic media and this iron may remain in solution under conditions inhibitory to solution of inorganic forms of iron due to the extremely low ionization of the iron in organic compounds. Oxidation of iron may or may not result in precipitation. Precipitation of ferric iron does not necessarily indicate an immediately preceding oxidation. Upon aerobic exposure of solutions containing iron dissolved and reduced under anaerobic conditions oxidation takes place and precipitation may occur; these changes may be independent of microbial activity. Under aerobic conditions, iron introduced as ferrous sulfate in peptone solutions may become precipitated subsequent to microbial development; the amount of precipitation is correlated with the amount of ammonia formed as a result of the decomposition of the peptone. Precipitation of iron from organic compounds of the metal results from the decomposition of the organic radicles creating a greater abundance of ions of iron than existed in the original solution and more such ions than would create a saturation. Such precipitation is dependent upon activity of organisms capable of decomposing the organic radicles and not upon direct action of the microorganisms upon the iron.

26. *An Acid-Tolerant Bacterium Causing the Rotting of Silage.* E. G. Hastings and Harriet L. Mansfield, University of Wisconsin, Madison, Wis.

From silage in which rapid spoiling had taken place, but in which no mold was found, an organism was isolated which grew rapidly on silage (self-sterilized through long storage) reducing the acidity. This organism is a small aerobic rod. It grows rapidly on agar acidified with the following organic acids: formic, acetic, butyric, lactic, citric, malic, tartaric and succinic, causing a reduction of acidity within twenty-four hours. Agar acidified with HCl and H_2SO_4 to a pH of about 4.0 supported growth, and the acidity was reduced in fourteen hours. This points to the possibility of neutralization in the case of organic acids, rather than destruction of the acid radical. Sauerkraut was inoculated with a suspension of the organism, but no growth was obtained. This may have been due to the salt concentration which amounts to about 2.5 per cent. This concentration of salt in acid agar and in silage inhibits the growth of the organism.

27. *Gassy Fermentation in Processed Swiss Cheese Containing Pimentos.* W. R. ALBUS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

This gassy fermentation was found to be caused by anaerobic gas-producing bacilli which develop because of the carbohydrate supplied by the pimentos.

28. *The Specific Resistance of Bacteria to Soap Solutions.* S. C. PRESCOTT AND P. L. RILEY, Massachusetts Institute of Technology, Cambridge, Mass.

During some tests to determine the germicidal power of certain soaps, it was noted that the different soaps, under identical conditions, had different germicidal strengths. It was also noted that any one soap, under the same conditions, was apparently more effective against one organism than another. This observation lead to the belief that the germicidal effect of the soap may be modified by the "specific resistance" of the organism.

With this thought in mind we studied the specific resistance of certain bacteria when exposed to soap solutions under uniform conditions of temperature, concentration, and time of exposure.

The results of the investigation show a pronounced specific resistance for each organism and even of different strains of the same organism.

29. *Preliminary Studies on the Microbiology of Manila Hemp.* P. K. Bates and S. C. Prescott, Massachusetts Institute of Technology, Cambridge, Mass.

The microörganisms found on manila hemp consist chiefly of several types of large bacilli together with a few molds,—aspergilli. The bacilli are unusually large, quick growing, spore formers. When inoculated on ordinary laboratory media they grow most luxuriantly on potato slants and starch agar, but growth was found on practically all media. Nitrate broth was vigorously reduced to nitrite ammonia. Pellicles were formed on glucose and other sugar broths, but no fermentative changes were observed. Peptonization of milk and liquefaction of gelatin were very marked in some cases.

The best grade of fibre shows few types and smaller numbers of organisms, while poor grades are highly infected. Both the types and the numbers of organisms are decreased when the fibres are held in ordinary dry storage. Samples of fibres from the same bundles were usually found to have one type of bacillus predominating.

Tests covering an incubation period of six weeks showed slight increase in numbers of organisms but no marked decrease in strength of fibre. Some evidence was found to indicate that the organisms digest or weaken the cement substances binding the fibres together.

The molds develop comparatively rapidly on the fibres when kept in a moist atmosphere, and may attack both the gum and the fibre substance itself. Work in this field is to be continued.

30. *Studies on the Bacteriology of Rubber Latex.* S. C. Prescott and W. E. P. Doelger, Massachusetts Institute of Technology, Cambridge, Mass.

Evidence has accumulated to indicate that bacteria may have a marked effect on the keeping quality and behavior of rubber latex, which is a fluid of great instability. On examination a large number of bacteria were found, some of which were without apparent effect on the latex, while others were especially active. The latex bacteria were invariably strongly acid producing when grown in the presence of carbohydrates. These organisms when grown in ammonia free sterile latex produce a putrefactive odor and a coagulation similar to the coagulation of milk but require much longer periods of time. Addition of sugars, even in small amounts, greatly hastens the process and coagulation takes place in about twenty hours at 37°C., the globules of rubber coalescing to form a coagulum varying in character according to the

sugar used and the type of organism. No putrefactive odor is noticed under these conditions. The changes which these organisms produce simulate the changes in milk to a considerable degree. Since the non-acid forming varieties do not produce coagulation, it seems evident that the coagulation may be due to the formation of acid or to bacterial enzymes, or to the effect of the acid on some enzyme (coalase) naturally occurring in the latex itself.

Eleven types of bacteria were repeatedly found which may be regarded as characteristic of latex. Of these, four were of the strongly acid producing types. The coagulation may be prevented by the addition of ammonia which appears to inhibit the acid formers, although the organisms themselves are not destroyed. Nearly all the organisms found are spore forming and all the acid producing species are notably so. The organisms apparently gain access to the latex in the operation of collecting and preparation for shipping.

31. *The Reduction of Bacteria in Mountain Streams.* PAUL W. ALLEN, WM. E. COLE, AND GEO. M. CAMERON, Department of Bacteriology, University of Tennessee, Knoxville, Tenn.

Due to the lack of extensive data concerning the reduction of bacteria in mountain streams and due to the widespread belief that a flowing stream purifies itself every 7 miles, a stream purification laboratory has been established in the basement of Morrill Hall at the University of Tennessee. A stream bed 100 feet long and 14 inches wide with 4-inch side walls has been built and equipped with a motor driven Gould pump No. 2. With such an equipment and by the addition of stones and sand, and by tilting the 15 different sections of the stream bed it is possible to produce the conditions of water flowing in mountain streams. By changing the grade it is possible to produce any rate of flow per hour.

The average results obtained to date, approximate very closely an average bacterial reduction of 75 per cent when the stream is flowing 1 mile per hour for seven hours down a grade of 5 feet per 100 feet. These results hold for water containing approximately 1200 bacteria per cubic centimeter, with the agency of light eliminated.

32. *A Basic Factor in the Control of White Diarrhea.* PAUL W. ALLEN, WM. E. COLE, AND GEO. M. CAMERON, University of Tennessee, Knoxville, Tenn.

In the nation wide attempt to control infectious white diarrhea the

factor of soil contamination has not been given sufficient attention. In the results obtained in this investigation it has been found that the white diarrhea organisms live over from season to season in the infected soil of poultry yards.

Three different types of soil, rich black soil, clay loam and sand No. 80, show different rates of reduction of *Salmonella pullorum*.

SYMPOSIUM ON "TEACHING OF BACTERIOLOGY IN INSTITUTIONS OTHER THAN MEDICAL SCHOOLS"

1. *Method of Conducting a Course in General Bacteriology. D. H.* Bergey, School of Hygiene, University of Pennsylvania, Philadelphia, Pa.

The development of the subject of bacteriology for the beginner is believed to be accomplished most satisfactorily by introducing each new phase of the subject by: (1) a preliminary lecture, explanatory in nature; (2) by the outlining of a series of simple laboratory experiments for the student so as to bring out the phases of bacterial activity being studied; (3) by assigning suitable reading on the particular phase of the subject in different text books; (4) by trips to manufacturing plants where similar phases of bacterial activity may be observed on a large scale, and (5) by a conference or review with the class to ascertain whether all the students have obtained the desired information.

2. *Consideration of Methods in the Teaching of General Bacteriology.* Charles A. Hunter, State Health Laboratory, University of South Dakota, Vermilion, S. D.

(No abstract.)

3. *Teaching Bacteriology in a College of Liberal Arts.* Elizabeth F. Genung, Smith College, Northampton, Mass.

A new scheme is being worked out in Smith college, the purpose of which is to prepare students definitely for further training in Public Health fields. This consists in a definite group of courses in Chemistry, Zoology and Bacteriology for a student choosing this plan instead of the usual major in a single department. After the completion of her college course, the student is ready to pursue either a Medical course, a graduate course in a school of Public Health or to enter a laboratory.

This scheme presents certain important problems such as: The

number of courses in bacteriology which should be required; the content of these courses; the prerequisites for introductory and advanced courses; the amount of chemistry which should be required. The content of the courses involves many problems concerning not only the subject matter itself but the best methods of presenting it. Should there be lectures, discussions or recitations and what proportion of the time should be devoted to them? The content of the course as limited by the time at the instructors disposal is also a serious factor.

4. Bacteriology as a Cultural Subject. JEAN BROADHURST, Teachers College, Columbia University, New York City.

The special claims of bacteriology as a cultural subject are briefly, as follows: (1) The subject matter covers an enormous range: from the ultra microscopic cell to the sun; from the helpful organisms in the soil under our feet feeding upon nitrogen to the human disease organisms demanding hemoglobin for their food, etc. (2) The single *living* cell as the basic unit of living organisms is more nearly grasped in microbiology; in a short space of time we may study in many successive generations of individuals morphological variations, physiological processes, environmental effects including food relationships, etc., with a sequence of measurable changes showing that these living units must be in a highly plastic state—the one important condition of living protoplasm. (3) Our appreciation of and adaptation to the world we live in demands a general knowledge of bacteriology, including methods of disease transfer, the principles underlying disinfection and quarantine and such economic problems as our plant food supply, from production to spoilage and its prevention. (4) Bacteriology has many direct personal applications. The subject matter as taught does not necessarily grow more complicated as it becomes more varied, and as a "required science" it can almost be counted upon to arouse interest in students who "resist" physics or chemistry. (5) The history of bacteriology is a closely interwoven network of physics, chemistry and biology; its sequences are logical and marked by applications of importance, often of intense human interest. (6) Last, but not least, the laboratory work can be kept simple in character, so that the illustrative values are not lost in technic.

5. The Use of the Short Answer Type of Questions in Bacteriology Examinations. OSKAR HUNTER, George Washington University, Washington, D. C.

The grading of the traditional essay type of examination is both very

time-consuming and highly subjective and unreliable. The grades assigned by 142 teachers to one paper varied from 51 to 97, and the grades assigned by 114 teachers to another paper ranged from 28 to 91. It is also found that when a paper is reread by the same teacher after a lapse of six months or more the paper is not infrequently evaluated as much as 10 points higher or lower than on the former occasion.

Such being the handicaps of the traditional examination, it is not surprising that attempts should be made to devise an examination that yields the same results regardless of where it is given, by whom it is given, or when or by whom it is graded. Experiment shows that examinations cast on the short answer type of question most nearly fulfill these requirements. The short answer tests contain a large number of items, and permit a much wider sampling of the students' information than is true of the essay examination.

As the answers are controlled and are either definitely right or definitely wrong, bluffing is prevented, and at the same time all such extraneous factors as handwriting, spelling, grammatical errors, and other incidental things that seriously impair the validity of the essay type of examination are eliminated. The short answer tests are so constructed that the grading is purely objective, and a paper with 300 questions can be graded in 10 minutes with perfect accuracy by a clerk, even though he knows nothing of bacteriology.

In order to compare the relative validity of the short answer and the essay types of examination, both types were subjected to the following test in the George Washington University Medical School. The students were given a 3 hour examination consisting of 10 questions in the traditional essay form. A short answer examination which consisted of approximately 300 questions and which required one hour and a half to administer was also given. A practical examination was given in order to establish a criterion against which to check the validity of the other two types of examination. The correlation between the students' grades on the 3 hour essay examination and the scores on the practical test was only 0.24. But a correlation of 0.74 was secured between the scores on the short answer test and the scores on the practical test. These correlations indicate that as an instrument for determining an individual's knowledge of bacteriology the short answer test is a very valid and useful measuring device, and that the traditional examination indicated the relative ability of the students not very much better than would an alphabetical arrangement of their names.

THE PARA-TYPHOID B—SUIPESTIFER GROUP OF BACTERIA

STUDIES IN DIFFERENTIATION

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The present work is the outgrowth of a preliminary study by the authors of an organism which was first observed by Rettger as the etiological agent in very extensive and fatal epidemics of young ducklings in Connecticut and Massachusetts and which was described by Rettger and Scoville in 1920, and named by them *Bacterium anatum*, N.S. The losses in some instances were as high as 90 per cent of the respective broods.

This organism was first isolated from the blood of ducklings, and soon after from ovarian and abdominal cysts of breeding ducks. Two years later it was obtained in pure culture from the blood of diseased ducklings on three large duck farms on Long Island.

Bacterium anatum, as described by Rettger and Scoville, closely resembled *Bact. paratyphosum* B in its staining, morphological and cultural characters. It fermented glucose, levulose, galactose, maltose, arabinose, rhamnose, dextrin, inositol, dulcitol, and mannitol, with the production of both acid and gas. The action of the different strains on xylose was somewhat variable, though all attacked this substance at least to some degree. Lactose, sucrose, raffinose, inulin, adonitol and salicin were not affected. Simple agglutination tests showed the organism to cross-agglutinate with Para B (Schottmüller) to about one-half the titer of the serum.

The present authors (1924) found that *Bacterium anatum*

could be distinguished from *Bact. paratyphosum* B and *Bact. suipestifer* by the agglutinin absorption test. However, by this method the ten strains fell into two distinct serological groups, the strains of the one group failing to remove completely the agglutinins from antisera prepared from individuals in the other group. The two groups will for the sake of convenience and clarity be referred to as *Bact. anatum* A and *Bact. anatum* B.

These observations were in harmony with those made by Cooper and Krumwiede (1924) upon five of the strains originally isolated by Rettger. They related certain of the strains to the *Bact. pestis-caviae* type, while they regarded the others as constituting a type different from any of the other paratyphoids studied by them.

As all of the strains of *Bact. anatum* could be distinguished readily from *Bact. paratyphosum* A and *Bact. enteritidis* Gaertner, these were not included in the comparative studies, and observations were confined throughout the investigation to the *Bact. paratyphosum* B and *Bact. suipestifer* groups. For these comparative studies 60 different strains were collected from various sources. They were distributed as follows:

Bact. anatum, 10 (C_1, C_3, C_5, C_8, C_9, CW, OW, M_{22}, Tt and WX).
Bact. pestis-caviae, 5 (146, 320, APT_2, EC_6, MT II).
Canary para B, 9 (I to IX).
Para B, 15 (180, 244, 152, 151, 232, 234, 5, 8, 12, 31, 47, 149, 169, 175, AMS).
Para B, Type c, Heimann, 1(P). From Schütze.
Para B, Type G, Heimann, 1(G). From Schütze.
Para B, Type Newport, 1(N). From Schütze.
Para B, Type Reading. Strain Stanley, 1(S). From Schütze.
Para B, Type Reading. Strain Nicholls, 1(R). From Schütze.
Para B, Type abortus-equinus, 1(A). From Schütze.
Para B, Type Binns, 1(B). From Schütze.
Bact. suipestifer, 8(Mw, Ch, 171, 290, 6917, 7000, N. H. U.).
Hog cholera bacillus, 6(161, 16, 17, 18, 319, 350).

In this list the strains are grouped according to the names under which they were received. Many of them were incor-

rectly labeled, so that the grouping given here does not separate the organisms into their true serologic types.[1]

No attempt is made here to present a review of the literature on classification of the paratyphoids, and only occasional references are cited. For a detailed bibliography recent publications of Krumwiede and Jordan may be consulted.

The name "*Bacterium aertrycke*" is applied in this paper to the various animal paratyphoid organisms referred to by different authors as *Bact. pestis-caviae, Bact. typhi-murium* (II), *Bact. aertrycke*, mutton type, Rodent type, etc. All of these types, we believe, should be classified under one name. "*Bact. typhi-murium*" has priority, but it is a trinominal and its use has occasioned much confusion, due to the existence of two distinct organisms occurring under this name, the one an aertrycke-like type, and the other closely related to *Bact. enteritidis*. "*Bact. pestis-caviae*" is likewise a trinomial, and this name was not coined until 1908 when Wherry applied it to an organism isolated by Smith and Stewart from diseased guinea pigs in 1897. The name "*Bact. aertrycke*" was given by DeNobele in 1898 to the organism which he isolated from cases of meat poisoning.

The different Para B types which are indicated above as coming from Schütze constitute a group of apparently independent types described by Schütze (1920) and supplied by him to this laboratory.

FERMENTATION REACTIONS

Much emphasis has been placed by different workers on the value of fermentation reactions in the classification of the paratyphoid-enteritidis group. Ford (1905) suggested the use of arabinose to differentiate *Bact. suipestifer* from *Bact. enteritidis* and *Bact. paratyphosum* B; *Bact. suipestifer* failed to ferment this sugar, while Para B and *Bact. enteritidis* attacked it with the formation of acid and gas. Jordan (1917) and Krumwiede, Kohn and Valentine (1918) confirmed Ford's results. Weiss

[1] The reader is referred to the Ph.D. thesis of the senior author in the Yale University Library, for a full description of these 60 strains, and for a historical review and bibliography.

and Rice (1917) stated that *Bact. suipestifer* failed to ferment inositol, while *Bact. paratyphosum* B readily attacked this sugar. Their results have been substantiated by the work of Winslow, Kligler and Rothberg (1919) and Jordan (1923). Koser (1921) found that *Bact. suipestifer* failed to ferment trehalose, while the strains of the Para B group and *Bact. enteritidis* fermented it.

In this investigation the strains under study were examined for their ability to attack the following fermentable substances:

Hexoses:
- Glucose
- Levulose
- Galactose

Pentoses:
- Arabinose
- Xylose
- Rhamnose

Polysaccharides:
- Dextrin
- Inulin

Disaccharides:
- Lactose
- Maltose
- Sucrose
- Trehalose

Alcohols:
- Glycerol
- Mannitol
- Dulcitol
- Inositol
- Adonitol

Glucosides:
- Salicin

Trisaccharides:
- Raffinose
- Melezitose

In order to avoid any possible decomposition of the sugars by heat they were sterilized by Berkefeld filtration and added to the already sterilized broth. The fermentation reactions were recorded after twenty-four, forty-eight and seventy-two hours, and the tubes further examined for another week for gas production.

Gas formation was usually accompanied by marked acid production. When as much as 1 per cent of the fermentable substance was used the H ion concentration as a rule dropped to about pH 4.4 from the original 7.0. Glycerol was an exception. This was acted on only slightly by all of the strains.

Xylose was not acidified in three days by strains OW, CW, M22, Tt, Wx (all *Bact. anatum* B) and Mw (*Bact. suipestifer*). However, when they were incubated for five days all but Mw produced a distinct acidity in the medium. Strain Mw showed slight acid production by the tenth day.

Acid was formed by all of the strains from glucose, levulose, galactose, maltose, rhamnose, dextrin, xylose, mannitol and gly-

cerol. None attacked lactose, sucrose, raffinose, inulin or adonitol. Action upon arabinose, inositol, dulcitol and trehalose was variable and can be seen best in table 1. In this table the strains of *Bact. anatum* are given as a unit. The Para B group includes those strains whose serological characters relate them to

TABLE 1

Acid production

ORGANISM	NUMBER OF STRAINS	ACID PRODUCTION FROM			
		Arabinose	Inositol	Dulcitol	Trehalose
Bact. anatum......................	10	10+	10+	10+	10+
Para B group......................	36	36+	34+ 2−	36+	36+
Suipestifer group...................	14	9− 5+	9− 5+	9− 5+	9− 5+

\+ = acid production; − = no acid production.

TABLE 2

Gas production

	NUMBER OF STRAINS	GAS PRODUCTION FROM					
		Xylose	Rhamnose	Arabinose	Inositol	Dulcitol	Trehalose
Bact. anatum...........	10	5+ 5−	10+	10+	8+ 2−	10+	10+
Para B group...........	36	33+ 3−	33+ 3−	33+ 3−	28+ 8−	36+	33+ 3−
Suipestifer group........	14	14+	13+ 1−	13− 1+	14−	13− 1+	12− 2+

\+ = gas production; − = no gas production.

Bact. paratyphosum B and *Bact. aertrycke,* and those which are apparently antigenically independent. The *Bact. suipestifer* group includes the strains which appear to be more closely related serologically to *Bact. suipestifer* than to Para B and *Bact. aertrycke.*

Gas was produced by all strains from glucose, levulose, galactose, maltose, dextrin and mannitol. None was formed from lactose, sucrose, raffinose, inulin, adonitol and salicin. Gas production in xylose, rhamnose, arabinose, inositol, dulcitol and trehalose was variable, as seen in table 2.

The following strains failed to produce visible gas from xylose; OW, CW, M22, Tt, WX (*anatum*), Mw (*suipestifer*), 152 (Para B), and 161 (hog cholera). All of these produced acid, however.

In order to determine further the gas-producing ability of the different strains, the tryptic digest medium of Kulp (1924) was

TABLE 3

Gas production in casein digest, with and without yeast extract

ORGANISM	CASEIN DIGEST XYLOSE	CASEIN DIGEST YEAST EXTRACT (HEATED XYLOSE)	CASEIN DIGEST YEAST EXTRACT (FILTERED XYLOSE)	CASEIN DIGEST	CASEIN DIGEST YEAST EXTRACT (HEATED)	CASEIN DIGEST YEAST EXTRACT (FILTERED)	DIFCO PEPTON XYLOSE
	per cent	*per cent*	*per cent*				
OW	10	15	25	0	0	0	0
CW	10	15	20	0	0	0	0
M22	5	15	20	0	0	0	0
Tt	10	22	20	0	0	0	0
Wx	5	10	15	0	0	0	0
Mw	5	5	10	0	0	0	0
152	5	5	10	0	0	0	0
161	5	10	20	0	0	0	0
Para A	0	0	0	0	0	0	0

employed instead of Difco pepton. Yeast extract was added to the digest medium to obtain still more favorable conditions. The results obtained with this medium are shown in table 3.

It is seen here that strains which did not produce gas in xylose when grown in plain broth containing this pentose formed a measurable amount of gas from this sugar when a particularly favorable nitrogenous substrate was supplied. Casein digest xylose medium furnished the largest amounts of gas when it contained filtered yeast extract. *Bact. para-typhosum* A, carried as a control in the experiment, produced no gas in any of these special media.

Attempts to differentiate Para B and *Bact. suipestifer* types by the special medium (serum water and Andrade's indicator) of Krumwiede and his associates (1918) were unsuccessful.

Organisms of the Para B-suipestifer group show very marked differences in their fermentative properties: not only differences between the various sub groups, but often between strains within the same sub group. Even the employment of arabinose, dulcitol, inositol and trehalose which have been used by different investigators for this purpose, will not in itself mark a sharp division between the Para B and suipestifer types, particularly when large numbers of these organisms are employed. Some strains of *Bact. suipestifer* will be found to ferment one or more of these differential substances.

Of the fourteen strains of *Bact. suipestifer* examined here, five fermented inositol, arabinose, dulcitol and trehalose. Not all of the fourteen were typical strains, however. These five strains (319, P. G. U. and R) are also more or less irregular in their serological relationships, and none of them are fatal to rabbits when injected subcutaneously in small numbers, whereas typical suipestifer strains cause a fatal septicemia, as shown by Smith (1889).

All of the strains of the Para B group attacked arabinose, dulcitol and trehalose. Jordan (1923) found Para B strains to attack inositol regularly when acid production was used as an index. Two of the 36 strains employed here failed to attack inositol perceptibly. These two strains were also irregular in their serological properties They are two of the free-lance strains (N and S) described by Schütze (1920). Aside from their failure to attack inositol, they possess the fermentative properties of the Para B group.

All of the ten strains of *Bact. anatum* gave fermentative reactions typical of the Para B group.

Acid and gas production do not necessarily run parallel. Gas formation by the various strains which require a special nitrogenous base for gas development is variable and should not be relied upon as an index of fermentation.

The results obtained here indicate that fermentation reactions

cannot in themselves be relied upon to distinguish *Bact. paratyphosum* B and *Bact. aertrycke* from *Bact. suipestifer*. Negative results obtained with arabinose, dulcitol, inositol and trehalose are of decidedly more value than positive, for they would point to the absence of the Para B-aertrycke group. Very few strains of the Para B-aertrycke group fail to act upon these substances.

No known fermentation test or combination of fermentation tests is sufficient to differentiate the *Bact. suipestifer* group from the *Para B-aertrycke* types. However, a study of fermentation reactions may be of considerable value in confirming the results of serological and other tests.

AGGLUTINATION TESTS

In the course of the investigation antisera were prepared from several of the strains, and the ability of these sera to agglutinate the different strains was tested. For the preparation of the sera rabbits were injected subcutaneously, first with dead and then with living cultures of the bacilli. These injections were followed, except in one instance, by intravenous administration of living cultures. In the case of *Bact. suipestifer* this method could not be employed, since the administration of living organisms, even in small doses, causes a fatal septicemia. A killed carbolized suspension containing approximately five hundred million organisms per cubic centimeter was prepared and 1 cc. was injected daily for twenty-eight days. At the end of this time the anti-serum had an agglutinating titre of 4000.

The antigens used in the agglutination tests were prepared by washing off twenty-four-hour slant agar cultures of the organisms with carbolized saline and diluting to a density of 0.5 on the McFarland nephelometer scale. In setting up agglutination tests dilutions ranging from 1:400 to 1:16,000 were employed. The tubes were incubated forty-eight hours at 37 degrees, after the addition of the serum.

No difficulty was experienced through spontaneous agglutination except in the case of one "rough" variant which was encountered when a stock culture was plated.

A definite separation of all of the organisms under observation into types through simple agglutination is impossible. The strains give varied reactions with the different sera. In general we may say that a serum agglutinates strains of the homologous type in a higher dilution than strains of heterologous types.

First let us consider the group of *Bact. anatum* strains. Three of these strains, C1, C3 and C5, are agglutinated in much lower dilutions by the sera derived from strains C8, CW, OW, M22 and Wx than are the antigens prepared from these five strains as well as antigens prepared from Aertrycke strains. Also C1, C3 and C5 are agglutinated in much lower dilution by sera derived from strains of *Bact. aertrycke* (146 and 320) than are strains C8, C9, OW, CW, M22, Tt and Wx. In addition, a serum derived from strain Mw (Aertrycke) does not agglutinate strains C1, C3 and C5, while strains C8, C9, OW, CW, M22, Tt and Wx are agglutinated in high dilution. When we consider the action of serum derived from strain C3 differences are again brought out. This serum agglutinates strains C1, C3 and C5 very strongly, while the other strains of the anatum group are affected only in low dilutions. If agglutination tests may be considered as giving any indication of the nature of an organism, these differences seem to indicate that two types may be represented in the present collection of *Bact. anatum* strains, C1, C3 and C5 comprising one of these types, and C8, C9, OW, CW, M22, Tt and Wx the other. This assumption is borne out by further serologic tests.

Several other interesting facts are worthy of note. Strain 175, which Jordan (1923) found to be *Bact. paratyphosum* B, has in our hands proved to be only slightly susceptible to agglutination by any of the sera used in the tests. *Bact. aertrycke* Mw is a very interesting strain, as is also strain 152, a transplant of Mw carried in another laboratory for a number of years. These two strains are agglutinated by sera derived from *Bact. aertrycke* cultures and strains C8, OW, CW, M22, and Wx. Sera prepared from strains of *Bact. paratyphosum* B and *Bact. suipestifer* have no power to agglutinate these strains. Serum prepared from strain Mw agglutinates strains C8, C9, OW,

CW, M22, Tt, and Wx and strains of *Bact. aertrycke* in high dilution. The strains of the *Bact. paratyphosum* B type are acted upon only very slightly if at all. The *Bact. suipestifer* strains and the free lance types of Schütze are uninfluenced by this serum. In the light of receptor analyses lately made upon the Salmonella group by Andrewes (1922) and Krumwiede, Cooper and Provost (1924) the action of cultures Mw and 152 would indicate that they are deficient in group antigen. They are not affected by antisera derived from Para B (31) and *Bact. suipestifer* (17), although both of these sera are rich in group agglutinin. Evidently these two strains are highly specific.

Culture 319 (suipestifer) is also peculiar in its agglutinatory relations. This strain is the original "*B. paratyphosus* C" of Hirschfeld. It has been identified by Krumwiede (1916) and Ten Broeck (1920) as *Bact. suipestifer*. This strain fails to agglutinate with any of the sera used except those derived from *Bact. suipestifer* strains. These sera agglutinate the organism in high dilution. This culture has been tested several times at different periods and has invariably given the same reaction. Apparently it contains little group antigen.

Some of the organisms have shown marked differences in their agglutination at different periods. One such strain is *Bact. suipestifer* 18. When tested at one time it agglutinated in high dilution with serum derived from *Bact. paratyphosum* B 31. When tested at another time it was not agglutinated in any dilution by this serum. *Bact. suipestifer* 350 acted in like manner. *Bact. aertrycke* V also showed the same variation. The serum in question is known to contain large amounts of group agglutinin. Evidently then the group antigen, or rather the effective group antigen contained in these cultures, varied widely from time to time. These examples serve well to illustrate the instability of characters upon which cross agglutination depends, an instability which has been very noticeable during the course of this work. According to the writers' experience, when an organism is agglutinated by a serum of a different type, at different periods, it is likely that the highest dilution in which agglutination occurs will vary widely from time to time.

These marked variations in cross agglutination are probably explicable on the basis of type and group antigens contained in bacilli. It has been stated by Andrewes (1922) that the antigenic characters of the Salmonella group are extremely unstable, and that the antigenic properties of a culture can not be predicted at any time. Andrewes noted these differences only by isolating substrains, however. It is of extreme interest that a whole culture, carried along under as nearly uniform conditions as possible, may change its characters so completely. Here we apparently have an example of a stock culture changing from a "mixed" strain to a purely "type" strain. This naturally suggests the question—Can a stock culture become purely a "group" strain? If this were possible we would, no doubt, encounter strains which for a time at least possessed no demonstrable identity. Such a condition has been met with in this investigation, and will be discussed in a later paper.

While the ordinary agglutination test is obviously unsuitable as a final means of identifying the members of this group, it is often helpful in determining the relative position of a strain, and in pointing out to the worker where to look for its exact relations, which are to be determined by subsequent tests.

COMPLEMENT FIXATION

The complement fixation test has been applied to the differentiation of the paratyphoid group by several workers. Altmann (1910) and Sobernheim and Seligmann (1910) stated that *Bact. enteritidis* could be distinguished from *Bact. paratyphosum* B by this method. They were not successful in differentiating the different types of the Para B bacilli in this manner, however. Ten Broeck (1918) studied *Bact. suipestifer*, *Bact. aertrycke* and *Bact. paratyphosum* B by complement fixation. He found that *Bact. aertrycke* and *Bact. paratyphosum* B are indistinguishable by this test. The action of *Bact. suipestifer* was irregular.

The work done in the present investigation upon complement fixation was not extensive. It was carried out for the purpose

of determining the usefulness of the test in the differentiation of the types under study. Since the test did not prove to be as valuable as had been hoped, its use was not continued.

The test was carried out by following the technique of Rettger and White as closely as possible. Bacillary suspensions which were heated to 60°C. for thirty minutes, and which contained 0.5 per cent phenol and 1 per cent glycerol, were used as antigens.

One fact is forcibly brought out through the use of the complement fixation test. The strains of the *Bact. anatum* group are again divisible into two types. The first type, made up of strains C1, C3 and C5, when used as antigen fails to fix complement in the presence of immune serum derived from members of the second group which is composed of strains C8, C9, CW, OW, M22, Tt, and Wx. The first type also fails to react with serum derived from *Bact. paratyphosum* B and *Bact. aertrycke.*

The second type of *Bact. anatum* cannot be distinguished from *Bact. paratyphosum* B or *Bact. aertrycke* by the complement fixation test, nor can these two last-named organisms be distinguished from each other. Such differentiation is brought about only by the agglutinin absorption method.

AGGLUTININ ABSORPTION

In late years Castellani's absorption test has played a very prominent part in the classification of the Salmonellas. Boycott (1906) was one of the earliest workers to set apart *Bact. paratyphosum* B and *Bact. aertrycke* by its use. Bainbridge (1909) and O'Brien (1910) also recognized the possibilities of this test in the differentiation of species. In America it has been used with marked success for the differentiation of *Bact. suipestifer, Bact. aertrycke,* and *Bact. paratyphosum* B, particularly by Krumwiede and his associates, by Jordan and by Ten Broeck, all of whom have greatly increased our knowledge of the paratyphoid group through its use.

The agglutinin absorption test has been employed for the identification of the species of the paratyphoid-enteritidis group in two ways. The first of these consists in testing the ability of

individual strains to absorb agglutinins from various type antisera, and a given organism is considered identical with the type from whose serum it completely absorbs agglutinins. The principle of the second method lies in testing the ability of unknown strains to agglutinate with type sera from which the group agglutinins have been previously removed. Both of these methods have been widely tried and both have proved to be satisfactory for certain uses. It is impossible to state which method will yield the better results, for success is largely dependent upon what the worker desires to emphasize.

The first method is the more exact. The use of the individual strains as absorbing antigens to absorb the various type sera, afterward testing the ability of the absorbed serum to agglutinate the serum strain and the absorbing strain, gives an accurate index of the absorptive capacities of the unknown organisms. If the worker desires to determine as nearly as possible the antigenic nature of the various organisms, this device is of the greater value.

The second method may be used to divide the organisms into relatively broad types and to establish a tentative classification. One of the fallacies of this method is that it relies upon the absorption of common or group agglutinins from a type serum by an organism of a second type, leaving only the specific or type agglutinins in the serum active. The difficulty in such a procedure lies in the fact that not all strains of a heterologous type or types will remove the same amount of group agglutinin from a given strain. Let us suppose, for example, that an unknown organism (X) may be one of these three types, A, B or C. The interrelations between types A and B are closer than the relations between A and C or B and C. Granting that culture X in reality is identical to type B, the following condition might be encountered:

AGGLUTINATION WITH AGGLUTINATING ANTIGEN	AGGLUTINATION WITH SERUM TYPE A ABSORBED BY TYPE C ANTIGEN	AGGLUTINATION WITH SERUM TYPE B ABSORBED BY TYPE A ANTIGEN	AGGLUTINATION WITH SERUM TYPE C ABSORBED BY TYPE B ANTIGEN
X	+	+	–

Since strain X is similar to type B and more closely related to type A than is type C, it would agglutinate with type A serum from which agglutinins active against type C had been removed. Also, it would agglutinate with type B serum which had been subjected to absorption with organisms of type A. In such a case as this the worker would be unable to identify the type to which the organism belonged except by resorting to the method of using organism X as an absorbing antigen for sera A, B and C.

Nor is it necessary to include the possibility of three types being present, as in the above example. The same condition may exist when only two types are considered. The reason for these discrepancies is that two strains of the same type may not remove the same amount of group agglutinin from the serum of a second type. This condition arises from variation in the amount of group antigens present in different strains. Also it is difficult or impossible to draw a line between group characters and type characters. If we assume that, as Nicolle (1917) expresses it, a bacterium is composed of a mosaic of biological and antigenic characters and that these characters are strain specific and not species specific, which is undoubtedly true, it becomes evident that to assert that certain of these characters are common or group characters, while others are specific or type characters, is impossible.

Schütze (1921) and Krumwiede, Cooper and Provost (1925) have called attention to the dangers of using the absorption of common agglutinins alone as a basis for the identification of bacterial species, and have suggested the use of reciprocal absorption tests to identify species which are incapable of removing the agglutinin from any type sera used in the work of identification.

In this investigation the individual strains were used as antigens to absorb the type antisera. The antigens were prepared in the same manner as those used in the agglutination test, except that the suspensions were left undiluted. The amount of suspension necessary to remove agglutinins completely from the homologous serum was determined in the following manner: The organisms were washed from the surface of agar slants with

a small amount of carbolized saline solution, so as to furnish a dense suspension of the bacterial cells. The suspension was filtered through absorbent cotton in order to remove bacterial clumps and foreign suspended material. This antigen was divided into six portions and adjusted to different densities varying from 3.0 to 8.0 on the McFarland nephelometer scale by the addition of carbolized saline solution. Three and nine-tenths cubic centimeters of each dilution were placed in centrifuge tubes and 0.1 cc. of the homologous serum added to each tube. This made a 1:40 dilution of the serum. The tubes were incubated at 37° for two hours, with occasional shaking where sedimentation occurred. The antigen suspension containing the diluted serum was centrifuged to clearness and placed in a second centrifuge tube. Fresh organisms were then added from the surface of an agar slant until the required density was again reached. The tubes were then incubated at 37° for two hours, centrifuged and allowed to stand overnight in the icebox. The following morning the diluted serum was used to set up agglutination tests, the homologous organism being used as antigen. By following the above procedure it was possible to determine, within certain limits, the least amount of bacterial antigen necessary completely to absorb the agglutinins from the homologous serum. This amount was taken as the absorbing dose for the serum. The amount of antigen used in the identification of unknown strains was slightly in excess of the absorbing dose. After absorption the serum was tested for its ability to agglutinate the absorbing strain and the serum strain. These agglutinations were set up with dilutions covering the original serum range and extending as low as 2.5 per cent of the original titre of the serum. It is of utmost importance that the entire serum range be covered. At times pre-zone phenomena are met with and if only a single dilution is used to test for agglutination a false result may be obtained.

In most instances the organisms used here completely removed agglutinins from the serum of one type and left the others unaffected. The majority of the strains fall into three types. They absorb agglutinins from either *Bact. paratyphosum* B, *Bact.*

aertrycke or *Bact. suipestifer* serum. The strains falling into these three groups are as follows:

Bact. suipestifer: 17, 18, 171, 290, 6917, 7000, NH (all labelled suipestifer or hog cholera); P (type C. Heimann), and G (Type G, Schütze).

Bact. paratyphosum B: 31, 232, 234, 5, 8, 12, 47, 149, 169, and A. M. S. (all labelled para B.).

Bact. aertrycke: C8, C9, OW, CW, M22, Tt, and Wx (all anatum); MT II, 146, 320, and APT_2 (all pestis-caviae); I, II, III, IV, V, VI, VII, VIII, and IX (all canary); 161 (hog cholera); 180, 244, and 152 (para B), and Mw (suipestifer).

The last two strains, 152 and Mw, deserve special mention. They came originally from the same parent stock. Although Mw does not remove agglutinins from 146 antiserum, 146, as well as the other strains of the aertrycke group, removes agglutinins from Mw antiserum. Strain 152 also removes agglutinins from Mw antiserum, but does not remove them from 146 antiserum.

In contrast to the strains listed above, there are several strains which fail completely to remove agglutinins from any of the type sera. These are C1, C3 and C5 (anatum); EC_6 (pestis caviae); 151 and 175 (para B); Ch and U (suipestifer); N (Newport), S (Stanley), R (Reading), A (abortus-equinus), B (Binns), 16, 319; and 350 (hog cholera). Strains C1, C3 and C5 (anatum) apparently constitute a distinct and uniform group. These three strains completely remove the agglutinins from C3 antiserum, while the other strains (including anatum) all fail to do this. The unplaced strains were subjected to further absorption tests, two to three times the amount of antigen required to saturate the sera completely being used. The results of these tests were identical with those of the former. In order to determine as nearly as possible the relationships of these strains the following experiment was performed.

Bact. aertrycke 146 antiserum was absorbed by *Bact. paratyphosum B* 31 in order to remove group agglutinin. *Bact. paratyphosum* B 31 serum and *Bact. suipestifer* 17 serum were absorbed with *Bact. aertrycke* 146 to furnish sera which would

be type-specific. The cultures to be tested were then agglutinated against the three type sera. The results of the tests are given in table 4.

From the protocol in table 4 it would seem that strain EC6 belongs to the *Bact. aertrycke* group, while strains U, R, 16, 319, and 350 belong to the *Bact. suipestifer* group, or are closely related to this type. Strains C1, C3 and C5 (anatum), Ch, 151 and 175 (para B), N (Newport), S (Stanley), A (abortus), and B

TABLE 4

ANTIGEN	HIGHEST DILUTION IN WHICH AGGLUTINATION OCCURRED WITH		
	Suipestifer (17) absorbed by 146	Para B (31) absorbed by 146	Aertrycke (146) absorbed by 31
C1	0	0	0
C3	0	0	0
C5	0	0	0
EC6	0	0	4,000
Ch	0	0	0
151	0	0	0
175	0	0	0
U	4,000	0	0
N	0	0	0
S	0	0	0
R	2,000	0	0
A	0	0	0
B	0	0	0
16	4,000	0	0
319	4,000	0	0
350	4,000	0	0

0 indicates no agglutination at 1–100.

(Binns) give no agglutination with any of the absorbed sera. Strain 175 has proved to be only very slightly agglutinable in our hands. Jordan (1923) classified this strain as *Bact. paratyphosum* B. According to Schütze (1920) a poorly agglutinating strain is usually a poorly absorbing strain. This is probably the reason that we have been unable to identify this strain by the absorption test.

The results of the agglutinin absorption test emphasize the differences found in the *Bact. anatum* strains by simple agglu-

tination and complement fixation. It is apparent that seven of the strains are absorptively indistinguishable from *Bact. aertrycke*, and if the nature of an organism may be determined by the absorption test, these seven strains are typical *Bact. aertrycke*. The remaining three strains of the *Bact. anatum* group are evidently identical with each other and are of a distinct type. Their serological behavior sets them apart from the other types studied here. Their agglutination reactions with the other types are irregular and not all all like those of the closely related *Bact. paratyphosum* B and *Bact. aertrycke* groups. They do not cross-fix complement with either *Bact. paratyphosum* B or *Bact. aertrycke*. Unlike *Bact. suipestifer*, they are not fatal to rabbits upon injection. By the absorption test they do not display any great likeness to the types described by Schütze. These three strains of *Bact. anatum* compose a distinct and well defined antigenic type. For the present they may be referred to as anatum A to distinguish them from the other seven *Bact. anatum* strains, which appear to fall within the aertrycke type, and may for the time be referred to as anatum B (aertrycke type). Strain Ch (Chesterfield strain of Peck and Thompson, 1911) we have found to be antigenically distinct. It does not absorb agglutinins from any of the sera studied. The agglutinins contained in antiserum derived from strain Ch were not absorbed by any of the strains under observation.

The *Bact. paratyphosum* B group, as represented here, seems to be a very homogeneous group, no difference beingobservable in the individuals of this type. The *Bact. aertrycke* group shows some variation from the central type however. Culture Mw and its counterpart 152 are not absolutely typical Aertrycke strains. Neither of them removed agglutinins from serum derived from *Bact. aertrycke* 146 or from *Bact. anatum* OW. Both of these latter strains completely remove agglutinins from serum derived from Mw, as does 152. These are evidently the same sort of strains which Schütze (1921) designates as substrains. They are from the same parent stock and have been maintained in different laboratories for ten years. The identity of characters possessed by the two strains today is striking evidence of the permanence of paratyphoid types.

Strain EC6 is also complicated in its absorptive characters. It fails to absorb agglutinins from *Bact. aertrycke* 146 anti-serum and *Bact. aertrycke* Mw serum. However, it is agglutinated in high dilution by 146 serum which has been previously absorbed with *Bact. paratyphosum* B. No anti-serum has been prepared from this strain, so that is it impossible to judge whether it is antigenically distinct. If it is an antigenically deficient strain it is of lower altitude in the type than is strain Mw, as evidenced by its failure to remove agglutinins from Mw serum. We have designated this strain as being closely related to *Bact. aertrycke*. A definite statement as to whether it is *Bact. aertrycke* would not be justified on the grounds of a group absorption test.

The strains of the *Bact. suipestifer* group are apparently quite varied in their absorptive capacities. This agrees with the observation of Andrewes and Neave (1921). Of fourteen strains which seem to be rather closely related to *Bact. suipestifer* only nine completely absorb agglutinins from the type serum, even though the numbers of organisms used to absorb the serum be doubled.

Strain 350 (hog cholera), although it fails to absorb agglutinins from antiserum derived from *Bact. suipestifer* 17, is in every other way a typical suipestifer strain. It does not ferment arabinose, trehalose, dulcitol, or inositol. It has proved to be fatal to rabbits following subcutaneous injection and is in every other respect a counterpart of the organism chosen as the type strain. It is probably of a lower altitude in the type than is 17 (hog cholera), the culture used to prepare type antiserum. This view is strengthened by the fact that strain 319 (Hirschfeld) fails to remove agglutinins from the serum derived from 17. Krumwiede (1918) and Ten Broeck (1920a) have both reported that strain 319 removed agglutinins from serum prepared by the injection of strain 350. Thus it can be seen that strain 17 is of a relatively high altitude within the type and that 350 and 319 contain, as Schütze expresses it, less "effective agglutinatory antigen" than does strain 17.

This condition, as we see it, is one of the limitations of the agglutinin absorption test. It shows clearly that, although a

culture may fail to remove agglutinins from a type serum, it may still be a member of that type. This fact necessitates either the performance of a reciprocal absorption test or a group absorption test. The uncorrelated results of a negative absorption may not always be relied upon in fixing the identity of a bacillus.

Strain 16 (hog cholera) seems to stand in the same relation to the type strain as does 350. It is a typical hog cholera bacillus but does not completely remove agglutinins from 17 antiserum. Strains P and G in our hands give results which indicate that at least antigenically they are typical *Bact. suipestifer*. These two strains ferment inositol, dulcitol, trehalose, and arabinose. They act upon these fermentable substances only very slightly, however, never producing the amount of acidity formed by an actively fermenting strain. They are not fatal to rabbits upon injection in small numbers. They have shown the same characters as are possessed by "*B. paratyphosus* C" of Hirschfeld, represented here as strain 319. Since Hirschfeld's bacillus is generally acknowledged to be a strain of *Bact. suipestifer*, there seems to be no reason why strains P and G should not also be considered as *Bact. suipestifer*. Strain P is the "*B. paratyphosus* C" of Heinmann, while strain G is a representative of type G of Schütze (1920).

Strain U has the same weak action upon trehalose, arabinose, inositol and dulcitol as have strains P and G. It does not completely absorb the agglutinins of 17 serum, but does agglutinate with 17 serum which has been absorbed with *Bact. aertrycke*. Dr. Schütze[2] considers it very closely related to strains P and G. We have tentatively placed it in the *Bact. suipestifer* group.

Strain R is the fourth strain received from Dr. Schütze which seems to bear a close resemblance to *Bact. suipestifer*. It readily ferments the differential sugars and fails to absorb agglutinins from 17, the type serum. It agglutinates in fairly high dilution with 17 serum from which the group agglutinin has been removed by absorption with *Bact. aertrycke*. This would indicate

[2] Personal communication.

that it is more closely related to *Bact. suipestifer* than to *Bact. aertrycke.*

The writers have been unable to correlate the other cultures representing the types of Schütze, namely A, B, N and S, with any of the main types within the group. Apparently they are quite independent antigenically.

SUMMARY AND CONCLUSIONS

Sixty strains of the *Bact. paratyphosum* B—*Bact. suipestifer* group have been studied with regard to their fermentative reactions, agglutination, complement fixation, and agglutinin absorption.

The present collection of *Bact. anatum* strains is divisible into two types by the serological tests. One of these types is serologically identical with *Bact. aertrycke.* The other type does not correspond to any of the paratyphoid organisms with which comparison has been made.

The fermentation reactions of the entire group have been studied and found to be somewhat variable. *Bact. suipestifer* can usually be distinguished from the "B types," by its failure to ferment arabinose, trehalose, dulcitol, and inositol. Some strains of this type do ferment these substances, however, though quite slowly. *Bact. paratyphosum* B and *Bact. aertrycke* are identical in their action upon the fermentable substances studied.

Cross agglutination in the Para B-suipestifer group has been found to be very irregular. This may, in some cases, be due to the varying amounts of type and group antigens present in the various strains.

Complement fixation is apparently more specific than simple agglutination. By the use of the fixation test the two types of *Bact. anatum* can be clearly distinguished, but *Bact. aertrycke* and *Bact. paratyphosum* B cannot be differentiated.

By agglutinin absorption it is possible to divide this group into several types. Three main types are established in this way: *Bact. suipestifer, Bact. paratyphosum* B and *Bact. aertrycke.* Besides these three types there are apparently several independent strains. These are the abortus-equinus, Binns, Newport,

and Stanley types of Schütze (1920) and the independent anatum type (C_1C_3 and C_5). Strain Ch (Chesterfield strain of Peck and Thompson) has in our hands proved to be an antigenically distinct type. It seems to stand in the same relation to the remainder of the group as do the independent types of Schütze. Type G of Schütze (1920), as well as *Bact. paratyphosum* C of Heimann and *Bact. suipestifer* of Uhlenhuth, have proved to be very closely related to, if not identical with, *Bact. suipestifer.* The Reading type also seems closely allied to this type.

Some of the strains fall into one type when their fermentative characters are considered, and into another type on the basis of their serological affinities. Such strains are 319, R, P, G and U. These strains are not fatal to rabbits upon injection, and they ferment trehalose, dulcitol, arabinose, and inositol. These characters would indicate that they belong to the "B types." However, by agglutinin absorption and agglutination with absorbed sera they fall into the *Bact. suipestifer* group. Where these intermediate strains should be classified is a perplexing question. They have been tentatively placed in the suipestifer group on the basis of their serologic reactions.

Certain limitations of the absorption test have been set forth. Some strains have proved to be incapable of absorbing agglutinins from the type serum of the type to which they belong, so that mutual absorption is necessary to fix their identity absolutely. The test in some instances seems to be strain specific rather than type specific.

Some strains seem to exhibit an apparently permanent deficiency of group antigens. Others may at times become deficient in type antigens, so that only group strains may be isolated from them at this time.

While it seems possible to classify the Salmonella group by the use of the tests employed in this work, there must of necessity be some limitations placed upon such a classification. The use of agglutinin absorption and agglutination with absorbed sera, correlated where possible with fermentative reactions, seems to be the most logical method for the differentiation of these closely allied species. The absorption test, like any other biological

method, is not without its limitations. The greatest limitation of this test for purposes of classification seems to be its exceeding specificity. By this method we may differentiate not only single types, but single strains. These facts must be kept constantly in mind when applying the absorption test. The bacilli of this group have been shown to be to some extent variable. This variability is not taken into account by the absorption method, so that some strains may at times fail to remove agglutinins from the serum of the type to which they belong.

Also, types which are not absorptively identical with the major types of the group are found from time to time. and some provision must be made to take care of such types.

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STUDIES IN MICROBIC HEREDITY

XII. MICROBIC DISSOCIATION IN VIVO AS ILLUSTRATED BY A CASE OF SUBACUTE SEPTICOPYEMIA

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INTRODUCTION

It is our purpose to describe in this paper the bacteriologic evidence for a life cycle change *in vivo* that involves bacillary-coccus transformations, together with several intermediate biotypes. Although a similar case dealing with a fusospirillary complex was reported by us (Mellon, 1919) we were not at that time certain that the mechanism of bacillary-coccus transformations involved the coccoid exospores (zygospores?). Shortly after this we demonstrated to our satisfaction that these exospores really served as transition *anlagen* for such cyclic transformations (Mellon, 1920). Moreover the first visible changes in the coccoid exospore involved the appearance of two segmentation lines at right angles which give us a form of tetrad.

When transplanted, these tetrads reproduced as such and also as diplococci, although by varying the environment one or the other phase became dominant. We have reported this observation *in vitro* so frequently with different organisms that there remains in our mind no doubt of its validity, but previous to our study of this case we had observed none where we were permitted to trace so completely changes that paralleled those that occurred *in vitro*. This is our justification for a report on the rather unique findings in this case, which constitutes the last of the present series of studies.

SOURCE OF CULTURES AND CASE HISTORY

The case was admitted to the hospital on July 11, 1921, with a diagnosis of acute cholecystitis. The gall-bladder was removed and it showed evidence of a mild cholecystitis, from which was isolated a member of the *B. coli* group. A few days later the patient developed a continued fever of obscure origin with moderate leucocytosis. Some weeks later he complained of difficulty in swallowing and his thyroid gland became tender and swollen.

The gland was punctured under aseptic conditions and pus obtained for bacteriologic examination. It was later incised and drained. The fever which was not intermittent went to 104° to 105° and an occasional abscess occurred in the skin. On October 19 the patient died and autopsy showed an acute purulent thyroiditis, an acute bronchopneumonia and metastatic abscesses in the liver and kidneys.

RESULTS OF CULTURAL STUDY

Direct examination of the pus from the thyroid disclosed a most pleomorphic picture. There were not only long, rather coarse threads suggestive of a streptothrix but large coccoid, yeast-like forms, both of which were being phagocyted by the pus cells (polymorphs) (fig. 1 at *a* and *b*). Also there were shorter bacillary, fusiform or diphtheroid forms present, in addition to true tetrads comparable in size with the spore-like coccoids (exospores).

The probable significance of such a complex we now know very well as indicated in Study V of this series (Mellon, 1926), and in a much earlier communication (Mellon, 1919–1920). It is simply that these exospores (zygospores?) are the transition phase in the life cycle change of bacillus to coccus and *vice versa*. As a result we may expect to isolate not only the stabilized types of the bacillary coccus complex but also certain intermediate sub-complexes that have a reversibility that is readily shown to be a function of environment.

On such a basis the following isolations of types is not sur-

prising, and we no longer believe that the multiplicity of types involved is the result of mixed infection as that term is conventionally employed. On an anaerobic agar slant, to which a few drops of controlled hydrocele fluid and 0.5 per cent maltose had been added, was sown some of the thyroid pus, obtained by puncture into a closed portion of the gland.

In from three to four days small discrete colonies were seen growing on a background of a delicate grey film. The tube had a foul odor recalling the growth of *B. fusiformis*. The grey film was composed of a curved fusiform or leptothrix-like organism (fig. 2). The discrete more opaque colonies were made up of long thread forms, many of which were fragmented into shorter rod forms, very pleomorphic in character. Large numbers of the coccoid exospores were found, resembling in size and contour those seen in the pus itself (fig. 3).

The general similarity in the cultural characters of these bacillary and thread forms made it probable that they were fairly closely related biotypes. After a few days at room temperature the now aerobically growing tube yielded some colonies of pleomorphic micrococci, and the thread form of the discrete colonies had fragmented into shorter diphtheroid-like forms.

When this same pus was planted in a shake culture of the same medium as above many colonies of micrococci appeared in a day or so in the upper zone of the agar. In the lower levels the colonies were smaller and were composed of large diplococci and tetrads. The variation in size was great and there was unequal segmentation of the pleomorphic forms. The reaction to Gram was amphophilic among the large forms and positive in the smaller forms. The micrococci in the upper layers of the tube were Gram-positive, while the thread forms of the other culture were Gram-negative except for the exospores which retained the Gram stain much longer.[1]

The segregation of tetrad reproduction and other aberrant

[1] This variability in reaction of the Gram stain is, as we have set forth in a previous paper (Mellon, 1919), a function of the cyclic changes of the organism and is in no sense a degenerative phenomenon. A parallel situation exists with the acid-fast organisms as we have frequently had occasion to observe.

division forms to the lower zones of the tube suggested the rôle of oxygen tension in the changed morphology. This was readily confirmed by growing the diplococci on a hydrocele agar slant under partial tension. The result was the same, viz., reversion to tetrad form (fig. 4) and this reversion could be reproduced from the cocci at will for as long as we tested the culture, a period of two or three years.

I have referred to the fact that the pus from the thyroid gland contained large coccoid bodies or exospores. As we have indicated in previous papers it is the transformation of these bodies to tetrads that really marks the transition from the bacillary phase to the coccus. The exospore under stimulus of a selective environment first clears in the center and segmentation lines proceed to the periphery of the cell at right angles to one another to give the appearance of the tetrad. Figure 5 shows degeneration lines just barely visible.

In this connection it is of interest that anaerobic slants of maltose hydrocele agar were conducive to the development of

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FIG. 1. SMEAR OF PUS FROM THE THYROID GLAND SHOWING AT *a* THE THREAD FORM, AT *b* THE YEAST-LIKE COCCOID EXPOSURE BEING PHAGOCYTED

Some bacillary forms at *c* are faintly seen in the plasma of this pus cell. Carbol thionin, × 1200.

FIG. 2. FUSIFORM-LIKE BACILLI FROM THYROID PUS GROWING AS A GREYISH FILM ON MALTOSE HYDROCELE AGAR SLANTS

Carbol thionin × 1200

FIG. 3. A PURE CULTURE OF THE ANAEROBIC BACILLARY TYPE ISOLATED FROM THE THYROID PUS ON MALTOSE HYDROCELE AGAR

Note the large number of coccoid exospores. Carbol thionin, × 1200

FIG. 4. TETRAD REVERSIONS OF THE DIPLOCOCCUS WHEN THE LATTER IS GROWN ON MALTOSE AGAR SLANTS AND THE TUBES ARE SEALED WITH WAX

Carbol thionin, × 1200

FIG. 5. SMEAR OF THYROID PUS SHOWING AT *a* AN EXOSPORE FORM IN WHICH CAN BE FAINTLY SEEN THE DOUBLE SEGMENTATION LINES OF EARLY TETRAD FORMATION

Carbol thionin, × 1200

FIG. 6. MIXED THREAD AND BACILLARY FORMS FROM DISCRETE COLONIES ON MALTOSE HYDROCELE AGAR. FROM THYROID PUS

Carbol thionin, × 1200

FIG. 7. THE RELATIVELY NON-PLEOMORPHIC ANAEROBE ISOLATED FROM THE RENAL PUS

Note absence of coccoid exospores. Carbol thionin, × 1200

Fig. 1

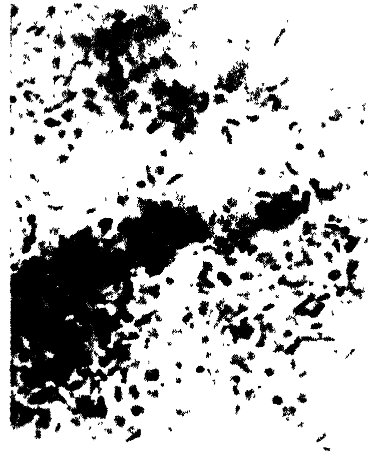

Fig. 3

Fig 2

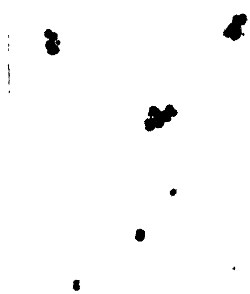

Fig 4

Fig 5

Fig. 6

Fig. 7

large numbers of the exospores—the parents of the cocci; yet under strict anaerobic conditions the cocci themselves refused to germinate on this medium. This indicates, of course, that the environment initiating an evolutionary change may not carry it to completion.

This principle was reported in our original observations on the mechanism of coccus development from "coccoids." Thus with our K. A. strain (Mellon, 1920) the maltose hydrocele mixture caused the initiation of tetrad development that only went to completion in a subsequent transplant when the maltose was withdrawn. Otherwise the abortive tetrads would never germinate.

THE CRUCIAL NATURE OF THE FINDINGS AT AUTOPSY

We were most fortunate to obtain an autopsy on this patient immediately after death. Miliary abscesses were found in both the liver and kidneys, which were examined aseptically. A most significant contrast was noted in the smears of the pus from these sources. The liver flora in its pleomorphic picture was not to be distinguished from that of the thyroid, *while the kidney pus showed only the filamentous forms: no exospores or tetrads were present.*

As a result it was possible to predict that no cocci would be isolated from the kidney on the ground that the exospores of the threads and bacilli represented their genetic origin; and from the liver we predicted as easily that cocci would be isolated because exospores and tetrad forms were present. The results confirmed the theory. A pure anaerobic flora *only* was obtained from the kidney, while from the liver the micrococcus and tetrad forms in addition were isolated. The latter grew readily under aerobic conditions at 37° and 22°C.

Therefore, in the absence of that pleomorphic phase in the life history of the bacillus in which exospores are produced *the micrococci can not develop*, and *vice versa*. It is obvious that some environmental factor in the kidney would not permit the production by the parasite of this special stage in its life history. This has its parallel *in vitro* for hydrocele maltose agar conduces to

exospore formation (fig. 3) while blood agar yields only the bacillary form.

Moreover the biotype isolated from the renal abscess was incapable of yielding exospores on maltose hydrocele agar. The contrast with the type isolated from the thyroid is noteworthy and harmonizes with the contention,—*no exospore, no tetrads or cocci.* Both of these types were strict anaerobes and had in general the same morphologic and cultural characters, differing however in their capacity for the formation of coccoid exospores.

Repeated attempts to isolate the coccus from the blood failed but the anaerobic thread form was obtained. Thus it is quite conceivable that the cocci of the liver abscesses may have been formed *in situ* from the thread form, assuming it to have been the primary infecting agent. Had they originated by metastasis from the thyroid via the blood stream their absence in the kidney would seem difficult of explanation, since this organ is especially vulnerable to hematogenous infections with this type of coccus.

After some months' cultivation the anaerobic thread forms showed a decreased ability to propagate under these conditions and gradually became adapted to partial tension conditions, but their morphology also became somewhat altered, the thread giving place to a bacillary form of the same general appearance.

At a later period we were able to cultivate this strain under aerobic conditions. Immune sera were developed in rabbits against this aerobic bacillary culture and the cocci. There was no evidence of cross agglutination nor of cross absorption in the sera. This is not surprising and, as we have repeatedly pointed out, is not final evidence for or against the genetic origin of the strains tested.

DISCUSSION

On the basis of previous studies of this sort I believe we are justified in regarding the infection in this instance as of the nature of an infecting complex—so to speak. Again, when one considers the rôle of filtrable phases of such organisms in the initiation of such infections (Mellon, 1926) it is difficult to say whether

any of the cultures isolated represented the primary infecting agent.

When one considers that the different morphologic types in blastomycosis are scarcely further removed from each other morphologically than are the distinct types isolated here, it is not necessary to invoke the mixed infection idea in cases of this sort. In fact, if it is used as the explanation, considerable difficulty will be experienced in reconciling all the facts observed, as has been indicated. The fact, too, that just such transformations have been repeatedly effected *in vitro* and in organisms isolated from such cases (Mellon, 1919 and 1926) justifies the point of view in this instance.

The coccus from the liver and the thyroid was culturally, morphologically and serologically the same. Its ability to revert to an unstable tetrad phase under certain conditions is atavistic evidence of its genetic origin, which has been referred to the exospores of the bacilli, whose evolution into tetrads we long ago demonstrated (Mellon, 1920).

From the fact that such dissociations take place in the host's tissues there is suggested a relation to immunity processes. It has recently been called to my attention by Hadley[2] that homologous serum is the most potent agent for effecting dissociation of virulent phases into non-virulent phases of the same organism, and that therefore this effect, rather than a germicidal one, may play an important rôle of immune serum. He suggests, too, that the "preparing" or opsonic action may be of this nature, and that the goal of much therapeutic endeavor might be directed to the use of substances possessing dissociation-furthering power even though they be not germicidal to any great extent.

Certain it is that the attack on microörganisms in the host must be made through the medium of the host's natural defenses rather than by direct attack on the invader. If the essays in chemotherapy have taught us anything it is this fact. For example, the illuminating work of the English observers (Dale and Dobell as I recall) has shown clearly that those emetin compounds

[2] Personal communication.

that kill amoeba in the test tube are worthless in experimental amoebiasis in the cat, while quite closely related compounds of the same drug will sterilize the cat's intestine but are harmless to the amoebae *in vitro!* The idea of *extermination* of any organism in the host is apt to be largely speculative.

CONCLUSIONS

1. In a case of suppurative thyroiditis and septicopyemia, aseptically obtained thyroid pus showed a bacterial flora of great pleomorphism.
2. The dominant form was a rather coarse thread or long bacillus which gave rise to coccoid exospores, and these in turn showed varying degrees of segmentation approaching closely a tetrad.
3. Miliary abscesses in the liver showed an identical flora but in the kidney the abscesses showed only thread forms with no exospores and tetrads.
4. Correspondingly, from the kidney only the anaerobic threads were obtained by culture, while from the liver and thyroid an aerobic micrococcus-tetrad culture was obtained in addition.
5. From previous *in vitro* demonstrations that tetrads and micrococci are genetically derived from the exospores of bacilli and threads the fundamentally composite nature of the flora of this case is explained.
6. The evidence is presumptive, therefore, that the kidney did not contain the coccus because it lacked the environmental essential that the liver and thyroid possessed, whereby the bacillus is forced through a complete life cycle that ends with coccus development.

GENERAL SUMMARY OF THE SERIES OF STUDIES ON MICROBIC HEREDITY

The aim of this series of studies, and some of the isolated ones preceding it, has been the demonstration that bacteria in their fundamental biology are in reality fungi that have been telescoped down, as it were, to a somewhat lower order; but this order is not so low as to preclude the preservation by the bacteria of the fundamental organization characterizing the fungi and higher

plants. In this respect they are entirely comparable to the internal organization of the atom which, despite its infinitely small size, is as much a solar system as is our own universe.

This point of view modifies considerably the first thing we learn about bacteria, viz., that they are schizomycetes in an absolute sense. In point of fact, instead of reproducing themselves by transverse fission alone, we know that under conditions none too specialized bacteria may bud, branch, form gonidia and even structures comparable with asci. The latter characteristic implies a complicated life cycle, sexual in nature. We believe that the evidence for such a sexual cycle is very considerable.

Not only have series of morphologic forms been encountered which, in composite, yield readily to the interpretation that they are stages in the process of zygospore formation, but the fate—genetically speaking—of these zygospores has been definitely determined. It is these structures—the so-called involution forms—that are the hereditary *anlagen* for the change of morphologic phase for which bacteria in their life cycles show marked capacity. The facts supporting this point of view practically abrogate the second thing we have been taught about bacteria, viz., the hypothesis that applies the term "involution" to forms entirely capable of germination *under suitable conditions.*

It is through the reorganization going on in these specialized structures that the appearance of new types within pure lines of bacteria is made possible. The latter may be closely related to the parent form or quite distantly removed in one or more characters. Thus, we have identified the process of bacterial variation with structures of known botanical significance, but whether the "variants" ultimately prove to be the result of mutation processes, or whether they are stabilized stages in the life history of the organism, or whether they are heterozygous in the Mendelian sense, may be left for the future to decide.

In any event it is clear, and now beyond dispute, that Koch's law of morphologic type specificity cannot longer be regarded in the absolute sense which has characterized it in the past. Bacteria do change their morphologic type and within the very widest

limits; and with this change may go at times important physiological modifications.

This subversion of the older point of view follows, logically enough, when we regard bacteria as having life cycles similar to those of the fungi and higher plants. This third point of fundamental difference with the older views requires a complete revaluation of the term pleomorphic. The conventional negative interpretation is now supplanted by a positive one which is essentially that of the mycologist. In other words bacterial polymorphism and the polymorphism of fungi both carry the implication of true life cycles.

The fact that bacteria pass through stages in their life history in which they are invisible and perhaps uncultivable is of much promise and interest from the standpoint of practical bacteriology. Thus, the question is raised whether many of our so-called viruses are not in reality invisible or filtrable stages in the life history of visible common microörganisms.

The interest in this point of view is intensified when we know that changes in virulence often accompany the changes in phase which the organisms undergo. This brings us into the practical domain of epidemiology, which still has so many problems virtually untouched. The point of view that the infecting phase or stage of a microörganism may have potentialities for virulence not possessed by the visible phase we have shown to be justified for organisms of the diphtheroid-fusiform complex. Thus it becomes possible that many of the organisms which we isolate, although incapable of producing disease in the stage in which we recover them, may nevertheless give rise to phases of their life cycles in which they are infective.

Thus certain common organisms associated with filtrable viruses may not always be regarded as secondary invaders. The concept of secondary infections in general may become modified in certain instances by a recognition of an infective complex, certain members of which may assume the rôle of producing complications of the primary disease.

Our serologic and diagnostic reactions are not unaffected by

these changed conceptions of the biology of bacteria. Serologic types are by no means absolutely fixed, and we know for a certainty that "variants" with "specific" agglutinogens may be split off from a "group" complex. The phenomena of inagglutinability as well as spontaneous agglutinability have been correlated in an experimental way with this new biology.

Our staining reactions also are affected. We can understand now how a Gram-positive organism may arise from a Gram-negative one, and we know that such changes take place. An organism, Gram-positive in one stage of its cycle, may become Gram-negative in another, without implying that a degeneration has occurred. The same is true for Gram amphophilism, a point often of importance in the identification of atypical meningococci. Precisely the same considerations hold for acid-fastness.

The fact then that our types of bacteria may only be stabilized stages in a complex may have important implications in many directions. Of interest to taxonomy and classification is the revised point of view. It seems clear that we are still far from knowing what a bacterial species actually is. In fact it is much easier to know what it is *not*. Certainly it is at present difficult to use the term in the same sense in which it is applied to the higher plants.

The writer has a keen realization of the fact that as an early pioneer in this disputed field many of the interpretations expressed in these studies will doubtless be further modified by the work of the future; but if the departure represented by his efforts is in the right general direction, and if it serves to galvanize into activity those of much greater biological insight than himself, he will have certain compensation for having shown what may have been considered an unwarranted temerity at a time when the tides of scientific thought all seemed to flow in the opposite direction.

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ESSENTIAL FOOD SUBSTANCES IN SOIL

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INTRODUCTION

Among the various external conditions for growth (Verworn, 1899) afforded by the environment, which are capable of acting as stimuli to the living cell, stimulating, accessory, or essential food substances are receiving a great deal of attention. Several of these are more or less well known chemically, while others are quite obscure.

Iron, iodine, phosphorus, potassium, manganese, boron, lignin xylose, alcohol, and nucleic acid, should be included in the list of essential food substances, for the action of these elements and compounds in certain concentrations is one of stimulation (Schreiner and Skinner, 1912; Warburg, 1914; Guilliermond, 1920; Thatcher, 1921; Warington, 1923; Cowdry, 1924; Gray and Chalmers, 1924; Puri, 1924).

In certain cases the true nature of these essential food substances is not clearly understood, and such terms as "vitamine," "bios," "auximones," "factors X and V," "X-substance," and "growth hormone," are found in the literature indicating the unknown factor of stimulation (Wildiers, 1901; Bottomley, 1917; Cole and Lloyd, 1917; Bachman, 1919; Williams, 1919; Robertson, 1921; Thjötta and Avery, 1921; Miller, 1924; Robertson, 1924).

Eastcott (1923) has shown that certain essential food substances are widely distributed in nature. Various plant materials are listed on the basis of the presence of one or both of the factors known as "Bios I" and "Bios II."

Bottomley (1917) and Mockeridge (1917) investigated "bac-

terized peat" and showed that this material contained essential food substances which they called "auximones." These substances supplied even in small quantities to *Lemma* plants growing in complete mineral culture solutions exerted a marked stimulating effect upon growth. These substances also influenced the nitrogen-fixing genera *Azotobacter* and *Rhizobium*, increasing nitrogen fixation and also the rate of nitrification by nitrifying soil bacteria (Mockeridge, 1917). These essential food substances were obtained from all the well-known and frequently used organic fertilizing materials. The greater the degree of bacterial decomposition which the material had undergone the greater was the proportion, as measured by their effect, of auximones. According to Mockeridge (1920) soil bacteria liberate from the soil organic matter, the growth-accessory or essential food factors which stimulate cellular activity. This effect may, of course, be due to products of microbial metabolism. The significance of this possibility is shown by Mockeridge (1924), who proved that *Azotobacter* and *Rhizobium* have a stimulating effect upon plant growth. A previous paper by the author, see Sanborn (1926), indicates that products of microbial metabolism may stimulate cellulose-destroyers in growth and physiological efficiency. It has also been shown that plant cells, e.g., leaves, seeds and seedlings, contain essential food substances which cause a similar stimulation. The seeds and seedlings investigated were those of alfalfa, barley and buckwheat.

The purpose of this experiment is to determine, in the light of Mockeridge's contention, if, during the decomposition of green manure, the essential food factor is elaborated and influences the cellulose-fermenting organisms in the ways indicated.

THE INFLUENCE EXERTED BY THE ESSENTIAL FOOD SUBSTANCES ELABORATED DURING THE DECOMPOSITION OF PLANT MATERIAL IN SOIL UPON THE GROWTH AND PHYSIOLOGICAL EFFICIENCY OF C. FOLIA

A poor soil (sandy loam) was prepared by mixing sand and loam, using equal parts of each. This soil was subjected to thorough

mixing in order to insure a homogeneous product. The crops to be investigated were grown in pots upon sandy loam. These were incubated at the proper greenhouse temperature with control pots. After the periods of growth indicated below, the crops were "plowed under," and allowed to decompose.

Examinations of the decompositions from time to time revealed, in a general way, the progress of the fermentation. When the processes appeared to be well on the way to completion, only a modicum of the skeletal remains being visible, 40 per cent aqueous extracts were prepared of all the soils including the controls.

CROP	AGE	NUMBER OF DAYS ALLOWED FOR DECOMPOSITION
	days	
Alfalfa	25	8
Barley	15	10
Buckwheat	17	8
Red clover	28	10

The aqueous suspensions of soil were filtered through sterile porcelain filters (Chamberland), and the filtrates tested for sterility by incubation and culturing upon beef-pepton agar. The sterile extracts were employed to test the influence of essential food substances elaborated during the decomposition of plant material in the soil, upon the growth and physiological efficiency of *C. folia.*[1]

The influence of these extracts upon the growth of *C. folia* was first tested. The extracts were added to a basic nutrient solution[2] making a total extract concentration equivalent to 0.4 gram of soil. A uniform inoculation with *C. folia*, approximately 6,000,000 cells, was given each flask, and the cultures were incubated at 27°. The results are recorded in table 1.

[1] *C. folia* is a new species, isolated by the author from decaying leaves, and described elsewhere.

[2] This nutrient medium is used throughout the investigation and is discussed elsewhere.

This table shows that the extracts prepared from the soils in which the crops had been plowed under and allowed to decompose, exert a marked stimulating influence upon the growth of *C. folia.* The control soil, which received no crop treatment, reveals negligible stimulation. In the nutrient medium alone there is a steady decline in bacterial numbers. It is evident that some essential food substance or substances must be operating in the extracts from the soils in which the crops decomposed.

TABLE 1

The influence of soil extracts, prepared from soils in which alfalfa, barley, buckwheat and clover have undergone decomposition, upon the growth of C. folia in basic nutrient solution

Expressed in millions

HOURS	CONTROL *C. folia* ALONE, NO EXTRACT	CONTROL EXTRACT FROM SOIL ALONE; NO CROP ADDED	EXTRACT FROM SOIL, ALFALFA DECOMPOSITION	EXTRACT FROM SOIL, BARLEY DECOMPOSITION	EXTRACT FROM SOIL, BUCKWHEAT DECOMPOSITION	EXTRACT FROM SOIL, CLOVER DECOMPOSITION
6	5.0	5 8	11 4	6.0	6.0	6 0
12	4.9	5.6	15.0	6.2	6.0	7 0
18	4 3	5 3	18.4	6.4	8.1	8.4
24	4.0	5 0	21.0	8.7	11.0	9 6
30	3.6	4 8	23.6	11.0	12.0	10.8
36	3.2	4.5	26.1	13.4	12.9	12.0
42	2.8	4.3	28.3	15.0	13.8	13 2
48	2.4	4.0	31.0	16 0	14.7	14 0
54	2.0	4.0	32.7	16 0	15.4	14 2
60	1.7	4.4	34.0	15 2	16.5	14.1
66	1.3	5.2	34.7	13.4	17.4	13 6
72	1.0	6.0	35.0	10.0	18.0	13 0

The influence of these substances upon the rate of cellulose decomposition by *C. folia* was next tested. Two-gram quantities of raw cotton were added to flasks of nutrient solution, and an extract concentration equivalent to 8 grams of soil was present in each 100 cc. of medium. Uniform inoculations from a young culture of *C. folia* in nutrient solution were made, of approximately 300,000,000 cells. The flasks were incubated at 27°. The electrometric and colorimetric methods for the determination of H-ion concentration were employed (Itano, 1923).

The rates of the decomposition processes were readily estimated in a preliminary experiment by visual observation of the macroscopic and microscopic changes: +++ = rapid; ++ = moderate; + = slow.

CROP	AFTER SEVEN DAYS FERMENTATION
Alfalfa	+++
Buckwheat	+++
Barley	++
Clover	++
Control	+

The quantitative results based upon H-ion concentration changes are given in table 2. These figures indicate that the stimulation is manifest early in the decomposition process, i.e., during the first two weeks. After that the accessory influence becomes less marked.

RÔLE OF THE ESSENTIAL FOOD FACTOR IN THE DECOMPOSITION OF GREEN MANURE

The results recorded above indicate that the acceleration in the rate of cellulose decomposition and the stimulation of the physiological efficiency of cellulose-destroyers takes place early in the process of destruction. Although in the decomposition of green manure the cellulose-destroyers eventually obtain essential food substances, and hence receive due stimulation, a preliminary dissolution of the cell wall of the plant must take place before the essential food substances are liberated. Of course, under normal conditions there is probably a supply of the essential food factor in the soil, so that the entire process is accelerated more or less without a definite lag period. Under abnormal conditions, some other factor such as moisture conditions or oxygen supply may dominate the decomposition process and the essential food factor becomes relatively less important. In this investigation. however, normal conditions prevailing in good soil are considered, and here the stimulation

in cellulose fermentation would seem to form a cycle in which cellulose-destroyers receive stimulation from present and past processes to attack fresh plant material, later liberating from this material the essential food factor, which is thus rendered available for other organisms. If this be true, it should be possible, by controlling the conditions of the experiment, to demonstrate this lag period, during which the medium is deficient in

TABLE 2

The influence of extracts prepared from soils in which alfalfa, barley, buckwheat, and clover have undergone decomposition upon the rate of cellulose fermentation by C. folia in a medium composed of nutrient solution and raw cotton in terms of pH

DAYS	CONTROL; EXTRACT FROM SOIL ALONE NO CROP ADDED	EXTRACT FROM SOIL, ALFALFA DECOMPOSITION	EXTRACT FROM SOIL, BUCKWHEAT DECOMPOSITION	EXTRACT FROM SOIL, BARLEY DECOMPOSITION	EXTRACT FROM SOIL, CLOVER DECOMPOSITION
Initial	8.40	8.40	8.40	8.40	8.40
1	8.23	8.04	8.00	8.10	8.16
2	8.07	7.68	7.54	7.76	7.92
3	7.90	7.30	7.12	7.44	7.68
4	7.74	6.94	6.72	7.12	7.44
5	7.57	6.65	6 44	6 82	7.20
6	7.40	6.34	6 26	6.62	6.94
7	7.24	6.18	6.15	6.49	6.70
8	7.06	6.06	6.04	6.40	6.54
9	6.95	6.00	5.92	6.36	6.42
10	6.86	5.98	5.80	6.34	6.32
11	6.82	5.94	5.70	6.31	6.27
12	6.80	5.92	5.40	6.29	6.22
13		5.88	5 31	6.26	6.18
14		5.86	5.20	6.24	6.16

essential food elements. The cellulose-destroyers will under such conditions, be without the accessory influence until they succeed in splitting the plant material sufficiently to liberate the essential food substances for themselves. The addition of the extracts containing this factor, to the medium, should result in aggressive decomposition from the first with no lag period.

Crops of approximately the same ages as before were grown upon sandy loam. The entire plant was then removed, washed

thoroughly in sterile water, and sterilized in $HgCl_2$ (1:1000) for three to four minutes. After copious washing in sterile water the plants, in small bundles of 3 grams each, were transferred under aseptic conditions to sterile quartz sand which had been previously ignited and washed with sulphuric acid. In transferring, the process of "plowing under" was simulated. The plants were inoculated with pure cultures of *C. folia* and *Act. colorata.*[3] Each pot received a uniform inoculation. The plant tissue was then treated with the extract, delivered from a sterile pipette.

In observing the influence of the extracts it was found that the greatest change in the plant material occurred during the first three or four days; after that period the control pots also showed marked stimulation, which according to the theory, was to be expected. In other words, in four days the cellulose-destroyers had liberated enough of the essential food factor from the plant cells to cause a very considerable increase in the rate of cellulose decomposition. In the presence of the extracts, however, the green manure was largely destroyed *during* this four-day interval.

From day to day detailed macroscopical and microscopical examinations of the plant tissues were made. The changes in the control pots were negligible until after the fourth day. In the majority of the pots which had received the extracts from the decomposed crops, the plant tissue was largely destroyed at the end of the third day of the fermentation. Of course, in the control pots the associative action of *Act. colorata* and *C. folia* might cause stimulation. As a matter of fact, activation from this source would probably not occur until later in the process. In a previous paper the author has shown that association with *Act. colorata* causes an increase in the cellulose-decomposing ability of *C. folia* at the expense of the former organism which gradually dies out. In the present case, the *Actinomyces* existed largely in the vegetative stage during the period of the experiment.

[3] *Act. colorata* is a new species and an active destroyer of cellulose; this organism was isolated by the author from decomposing leaves.

DISCUSSION

It is evident that some essential food factor is present in the plants under investigation. Whether this be of the nature of "Bios I" and "Bios II" suggested by Eastcott (1923), of Bottomley's auximones (1917), or some better known substances, such as a minute amount of carbohydrate or humate (Voicu, 1923), is uncertain.

However, in common with these other substances, extracts prepared from the above-mentioned crops exert a stimulating influence upon cellular activity. Microörganisms of the soil are so influenced.[4] The author, in a previous paper, has reported the stimulation of cellulose destroyers by such extracts. During the destruction of these crops in the soil, the essential food substance is elaborated into the surrounding medium, and stimulates cellulose-decomposing organisms in growth and cellulose-destroying ability.

These results are in accord with the contention of Mockeridge, who states that through the action of soil bacteria, the growth-accessory factor is liberated and stimulates the nitrogen-fixing genera *Azotobacter* and *Rhizobium*, and the nitrifying soil bacteria. Bottomley and Mockeridge find at least a partial explanation of this phenomenon in a study of nucleic acid and its derivatives.

GENERAL CONCLUSIONS

1. Essential food substances, present in alfalfa, barley, buckwheat and clover plants, are elaborated into the soil during the process of decomposition.

2. These substances are available for soil microörganisms, and function as growth-accessory or stimulating factors. Cellulose destroyers are stimulated both in growth and physiological efficiency.

[4] The influence of green manures upon the growth and physiological efficiency of *Azotobacter chroococcum*. A. Zoond. Thesis for degree of M.Sc., McGill University. Not yet published.

3. The stimulation in the physiological activity of cellulose-decomposing organisms is manifest during the early stages of the fermentation.

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DIFFERENTIATION OF INTESTINAL ORGANISMS BY MEANS OF SEMIFLUID SUGAR MEDIA[1]

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In a previous paper the fact was noted that the progress of *Escherichia coli* through broth contained in slender tubes could be followed with the unaided eye. It was also observed that each species of bacteria under observation tended to progress at a uniform and constant rate characteristic for the strain and species. The present study was undertaken to ascertain the characteristic rate of progress of the ordinary intestinal organisms, as well as any peculiarities of appearance or behavior which might be turned to account in isolating typhoid, paratyphoid, or dysentery organisms. It was found that the rate of progression in semifluid media had some differential value. A culture medium was then devised which contains certain sugars as mentioned below, and which is of semifluid consistency. This medium appeared to be of value in isolating and identifying the organisms ordinarily found in the intestine.

TECHNIQUE

Tubing used was ordinary glass tubing with very thin wall, outside diameter 3 to 5 mm. For following progression during periods longer than one day, 4-foot lengths kept at room temperature were used. In all other experiments, 8- to 12-inch lengths were used, incubated at 37°C.

MEDIA

Two different kinds of media were used. (1) Plain meat extract broth of pH 7.6. Motile and non-motile organisms pro-

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gressed equally well through this medium. (2) Semifluid medium, somewhat similar to that of Hitchens, consisting of meat extract broth plus 0.5 per cent dried shredded agar, 1 per cent lactose and 1 per cent sucrose. Non-motile organisms grew at the point of inoculation in this medium but did not progress.

It was found convenient to fill the tubes automatically in lots of 200 by the following method. Tubes were sealed at one end; the open ends were then immersed in medium contained in a half liter beaker. Beaker and tubes were then heated slowly to 25 pound pressure in a steam sterilizer and the steam allowed to escape rather rapidly through a pet-cock until the pressure sank to 10 pounds. The flame was then extinguished. When apparatus had cooled, the medium was forced into the tubes by atmospheric pressure. All tubes were inoculated 1 inch deep through the open end. The tube was either sealed or capped with a small test tube, and incubated in the horizontal position. Table 1 shows the rate of progression and appearance of cultures grown in tubes filled with plain broth as described.

When dysentery organisms are grown in plain broth at room temperature, gravity exerts an unmistakable influence. The Hiss organism inoculated into the higher end of a tube inclined at an angle of 45° progressed $18\frac{1}{2}$ inches over night. The tube was then reversed so that the organism had to go up hill in order to progress. No progression occurred during twenty-four hours. The same tube reversed a second, third and fourth time gave the same result. The Flexner and Shiga organisms acted similarly.

When grown in the incubator in semifluid medium, both typhoid and colon organisms grew horizontally or vertically, up or down, at the same rate, indiscriminately.

The colon organisms grew in lactose broth provided the caliber of the tube was more than 3 mm. If a fine capillary tube was used with diameter approximately 0.1 mm. or less, the progress of colon organisms was retarded or stopped, apparently from the mechanical effect of gas bubbles. Both colon and typhoid organisms grew satisfactorily through fine capillary tubes provided no sugar was present and hence no gas bubbles.

When it was found that *Escherichia coli* progressed through

TABLE 1

Rate of progression and appearance of plain broth cultures, intestinal organisms

NAME OF ORGANISMS	PROGRESS FIRST DAY	PROGRESS FIRST WEEK	PROGRESS FIRST MONTH	APPEARANCE OF GROWTH
	inches	*inches*	*inches*	
1. *Eberthella typhi* Barry	4¼	30½	31	Segmented streak
2. *Eberthella typhi* Flexner	5	12½	14	Broad dotted streak, some turbidity ahead
3. *Eberthella typhi* Hopkins	4½	32½	32½	Segmented streak, some turbidity ahead
4. *Eberthella typhi* Hubacher	6	31	31	Broad irregular streak, some turbidity ahead
5. *Eberthella typhi* Jackson	4	15	15	Broad dotted streak, some turbidity ahead
6. *Eberthella typhi* Queen's Hospital	8	22	24	Thin streak, some turbidity ahead
7. *Eberthella typhi* Rawlings	7	18	19½	Irregular gapped, some turbidity ahead
8. *Eberthella dysenteriae*	6	16*		Broad, transversely striated, gapped streak, no turbidity ahead
9. *Eberthella paradysenteriae* Flexner	2½	2¾	18*	Narrow smooth gapped streak, some turbidity ahead
10. *Eberthella paradysenteriae* Hiss	7½	17½	36	Narrow smooth gapped streak, some turbidity ahead
11. *Salmonella aertrycke*	12½	34	36	Broad streak mobile colonies ahead
12. *Salmonella enteritidis*	13	46*		Broad streak mobile colonies ahead
13. *Salmonella paratyphi*	16			Irregular streak, some turbidity ahead
14. *Salmonella schotmulleri*	8	25*		Streak, mobile colonies ahead
15. *Escherichia coli*	24	144	600	Thin smooth streak, mobile colonies ahead
16. *Aerobacter aerogenes*	7	23	23½	Streak only, no turbidity
17. *Aerobacter cloacexa*	14	46*		Broad streak, mobile colonies ahead
18. *Proteus vulgaris*	7	42*		Broad segmented streak, turbidity ahead
19. *Vibrio comma*	7	22½	30*	Scattered dotty deposit
20. *Staphylococcus albus*	4	30		No turbidity, streak pointed

*Progress possibly limited by fact that growth reached end of tube.

culture tubes more rapidly than *Eberthella typhi*, an experiment was devised to ascertain whether the more rapid organism could overtake and pass the other organism in the tube. When Eberthella was inoculated only 1 inch in advance of Escherichia, the gas forming organism passed the other and progressed entirely through the tube, liberating gas bubbles as it went. When, however, Eberthella was inoculated 2 inches or more in advance, Escherichia caused gas bubbles for a distance of only 2 inches beyond the point of inoculation, while the Eberthella apparently grew evenly through to the end of the tube.

TABLE 2

Distance traversed by ordinary organisms; also presence or absence of gas; semifluid medium

NAME OF ORGANISM	PROGRESSION FIRST 24 HOURS	FERMENTATION
	inches	
Eberthella typhi Flexner	4	–
Eberthella typhi Hopkins	4	–
Eberthella typhi Hubacher	4	–
Eberthella typhi Jackson	4	–
Eberthella typhi Rawlings	3	–
Eberthella dysenteriae Shiga	$\frac{1}{4}$	–
Eberthella paradysenteriae Flexner	$\frac{1}{4}$	–
Eberthella paradysenteriae "Y"	$\frac{1}{4}$	–
Salmonella paratyphi	10	–
Salmonella Schottmulleri	10	–
Salmonella aertrycke	10	–
Escherichia coli	10	⊕
Aerobacter aerogenes	3	⊕
Aerobacter cloacae	10	⊕
Proteus vulgaris	9	⊕

It was found that the addition of 0.5 per cent agar to the broth rendered it almost impermeable to the dysentery group and to non-motile organisms, while motile organisms progressed rapidly through it. A differentiating medium was then devised according to the formula given above. Lactose was of course used to facilitate the detection of Escherichia and other lactose splitters. Sucrose was added to reveal *Proteus vulgaris*, which does not split lactose. When stock strains of ordinary organisms were

used, the semifluid medium always enabled us to make a satisfactory differentiation. The colon bacillus rapidly progressed through the medium leaving the trail of gas bubbles. The dysentery organisms did not progress, but formed visible colonies at the site of inoculation. Typhoid (five strains), Paratyphoid A and Paratyphoid B all progressed through the medium but did not form gas. In practice, it is anticipated that only those strains which progress without forming gas in the sugar media will require agglutination against typhoid and paratyphoid sera. Table 2 shows characteristics of cultures of ordinary organisms when grown in semifluid medium. The semifluid medium was then used for the investigation of 422 colonies picked

TABLE 3

Characteristics of colonies picked from endo plates and inoculated into semifluid medium

AMOUNT OF PROGRESSION, 24 HOURS	GAS PRESENT	GAS ABSENT	TOTAL
Less than 1 inch	12	131	143
Between 1 and 2 inches	3	3	6
Between 2 and 4 inches	12	3	15
Between 4 and 6 inches	32	0	32
Between 6 and 8 inches	13	2	15
Over 8 inches	202	9	211
Total colonies examined	274	148	422

from Endo plates which had been streaked from the feces of 192 supposedly healthy individuals. Table 3 shows the amount of progression and the presence or absence of gas.

For opportunity to carry on the experiments, thanks are due to Col. Edward R. Schreiner, M.C., Commanding Officer, Tripler General Hospital, Honolulu, T. H. For counsel and assistance the author is indebted to Col. Charles F. Craig and Major Henry J. Nichols, both of Medical Corps, U. S. A. For valuable technical assistance acknowledgements are due First Lt. Mary F. McLaughlin, A.N.C., U. S. Army. For routine assistance, thanks are rendered Pvt. First Class Edgar H. Freeman, Med. Dept., U. S. Army.

SUMMARY AND CONCLUSIONS

1. The ordinary intestinal bacteria have been grown in plain broth and also in semifluid medium contained in slender glass tubes kept horizontally.

2. A characteristic rate of progression and appearance have been noted for each of the following organisms: Ten strains of three species of Eberthella; four species of Salmonella; *Escherichia coli;* two species of Aerobacter; *Proteus vulgaris; Vibrio comma; Staphylococcus albus.*

3. Slender tubes containing semifluid carbohydrate media have been used. Only typhoid and paratyphoid organisms (and a few other closely related forms) will progress through these tubes without forming gas. The use of these cultures renders many agglutinations unnecessary and thus facilitates recognition of typhoid and paratyphoid.

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CULTURAL AND ANTIGENIC STUDIES ON SALMONELLA GALLINARUM AND SALMONELLA PULLORUM[1]

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Since it has been shown by Gage (1914) and others that antigens prepared from different strains of *Salmonella pullorum* will give different results when used in testing for ovarian infection in adult fowls and since the senior author and his associates found this to be true to a very marked degree when certain *S. pullorum* strains were used, it appeared probable that separate antigenic groups might be found within this species. This work was, therefore, undertaken to establish the presence or absence of such groups, to help in establishing the position of *S. pullorum* and *S. gallinarum*, and to throw more light on the identification of these organisms as well as on the agglutination test for the identification of carriers of *S. pullorum*.

CULTURAL REACTIONS

The 83 cultures chosen for this study are listed in table 1 and information is given as to source, type of infections, original designation and previous descriptions.

The order of arrangement was established after a study of experimental data, and, in general, related strains are grouped together.

The organisms studied are all gram-negative, non-motile rods, varying considerably in size but being always less then 1μ in width and usually less than 2μ in length. Although some data

[1] Contribution No. 335 of the R. I. Agr. Exp. Sta.

TABLE 1

Strains of S. pullorum and S. gallinarum studied

NUMBER	SOURCE	REMARKS	ISOLATED OR RECEIVED
		S. pullorum	
12*	Gage, Mass. Agr. Coll.	Yolk of chick inoculated with strain 2	1915
13*	Gage, Mass. Agr. Coll.	Strain 5	1915
14*	Gage, Mass. Agr. Coll.	Strain 6	1915
15*	Gage, Mass. Agr. Coll.	Strain 25–1	1915
17*†	Hadley, R. I.	Chick lung	1910
18	Gage, Mass. Agr. Coll.	Strain 25–2. Chick yolk	1915
20	Rettger, Yale Univ.	Strain C–'16	1916
34*†	Hadley, R. I.	Chick lung	1914
99*	Hadley, R. I.	Chick lung	1914
123	Rettger, Yale Univ.	Strain "Cosgr."	1916
124	Rettger, Yale Univ.	Strain B–'15	1916
125	Rettger, Yale Univ.	Strain R–'15	1916
126	Gage, Mass. Agr. Coll.	Strain C–1	1916
127	Gage, Mass. Agr. Coll.	Strain L–1	1916
128	Gage, Mass. Agr. Coll.	Strain T–1	1916
129	Gage, Mass. Agr. Coll.	Strain W–1	1916
162*	Hadley, R. I.	Adult fowl	1917
167	Hadley, R. I.	Chick liver	1915
170	Hadley, R. I.	Chick heart	1916
220	Hadley, R. I.	Unabsorbed yolk	1920
223	May, R. I.	Chick heart	1921
224	May, R. I.	Chick heart	1921
225	May, R. I.	Chick heart	1921
226‡	May, R. I.	Chick heart	1921
227‡	May, R. I.	Chick heart	1921
228‡	May, R. I.	Chick heart	1921
229‡	May, R. I.	Chick heart	1921
230	May, R. I.	Chick heart	1921
257	May, R. I.	Yolk of egg in ovary	1924
258	May, R. I.	Liver of chick inoculated with 257	1924
272	May, R. I.	Heart of adult male	1924
277	May, R. I.	Chick liver	1925
19	Rettger, Yale Univ.	Strain W–'15	1916
102A*	Hadley, R. I.	Liver of adult fowl	1914
102B*	Hadley, R. I.	Heart, same fowl as 102A	1914
117	Hadley, R. I.	Chick heart	1915
118*	Hadley, R. I.	Adult fowl (N. Y.)	1915
122	Rettger, Yale Univ.	Strain A–'16	1916

TABLE 1—*Continued*

NUMBER	SOURCE	REMARKS	ISOLATED OR RECEIVED
		S. pullorum—continued	
165	Hadley, R. I.	Adult fowl	1916
168	Hadley, R. I.	Liver of adult fowl (Mass.)	1915
169	Hadley, R. I.	Chick heart	1915
224A	Tittsler, R. I.	Chick inoculated with 224	1923
223	May, R. I.	Chick heart	1922
234	May, R. I.	Chick liver	1922
244	May, R. I.	Chick heart. Same flock as 224	1923
245	May, R. I.	Heart of adult fowl	1923
246	May, R. I.	Chick liver. Same flock as 224	1924
247	May, R. I.	Chick heart	1924
271	May, R. I.	Heart, adult fowl inoculated with 102A	1924
273	May, R. I.	Yolk in ovary, fowl inoculated with 118	1924
275	May, R. I.	Liver, adult fowl, no epidemic	1924
276	May, R. I.	Chick lung	1925
		S. gallinarum	
38	Rettger, Yale Univ.	Originally Cornell	1915
110*	Hadley, R. I.	Liver of fowl	1914
115*	Th. Smith, Harvard	Original *Bact. sanguinarium* of Moore	1915
116*	Th. Smith, Harvard	Strain F-T II	1915
147	Gage, Mass. Agr. Coll.		1917
148	Gage, Mass. Agr. Coll.		1917
158*	Colo. St. Vet. Coll.	"B. avisepticus"	1917
161	Goldberg, Cornell	Isolated by Taylor, in California	1917
163	C. G. Bull, Rockefeller Inst.	"B. avisepticus"	1917
164	Murray, Iowa Sta. Coll.		1917
202	Kral's Laboratory	*Bact. gallinarum*, Klein, London	1919
206	Pasteur Inst., Paris	Seine and Marne	1920
207	Pasteur Inst., Paris	Aube	1920
208	Pasteur Inst., Paris	Ain	1920
209	Pasteur Inst., Paris	Cote Garonne	1920
210	Pasteur Inst., Paris	Loiret	1920
211	Pasteur Inst., Paris	Loiret 2	1920

TABLE 1—*Continued*

NUMBER	SOURCE	REMARKS	ISOLATED OR RECEIVED
		S. gallinarum—*continued*	
212	Pasteur Inst., Paris	Doubs	1920
213	Pasteur Inst., Paris	Aude	1920
214	Pasteur Inst., Paris	Cote Garonne	1920
215	Pasteur Inst., Paris	Yonne	1920
216	Pasteur Inst., Paris	Originally from U. S. Dept. of Agr.	1920
221	Hadley, R. I.	Spleen of fowl	1920
235	May, R. I.	Fowl liver	1922
236	May, R. I.	Heart, fowl, same flock	1922
237	May, R. I.	Fowl spleen	1922
45*	Hadley, R. I.	Fowl liver	1910
47*	Pasteur Inst., Paris	Bellefontaine Strain	1911
65*	Rettger, Yale Univ.	Strain "Simmons, 1910."	1912
66*	Rettger, Yale Univ.	Strain "New Haven, 1909"	1912
88*	Kral's Laboratory	Original *B. gallinarum* of Klein	1914

* Discussed by Hadley (1918).
† Discussed by Mulsow (1919).
‡ Chicks from same lot.

have been accumulated on the effect of drying, heat and chemicals, these throw no light on the grouping of the different strains and so are omitted from this paper. The reactions in litmus milk show no striking variation from the usual reactions of cultures of these groups, namely slight acid formation, followed by alkalinization and ultimately by saponification.

For the purpose of studying the fermentation of carbohydrates the organisms were grown in Dunham tubes of meat extract broth containing 1 per cent of the carbohydrate in question to which Andrade's indicator had been added. Readings were made at appropriate intervals as to the presence or absence of acid or gas up to fourteen days.

The results of these tests are summarized in table 2 and certain peculiarities are discussed in the text. For the sake of brevity this discussion takes up the carbohydrates in groups according to the type of reaction produced.

In arabinose, xylose, rhamnose, glucose, levulose, mannose, galactose and mannitol most strains of *S. gallinarum* and all strains of *S. pullorum* regularly produce acid, while the latter as a rule also produce gas. *S. gallinarum* No. 45 has occasionally failed to ferment xylose and 47 and 66 have consistently failed to do so. *S. gallinarum* also regularly produces acid from maltose, dextrin and dulcitol, while *S. pullorum* does not attack these substances. Lactose, sucrose, raffinose, inulin, erythritol and salicin are not fermented by either *S. pullorum* or *S. gallinarum.*

In the carbohydrates fermented by both species the production of acid is usually prompt, being strongly indicated in 24 hours. Xylose alone is an exception to this rule, as acid formation in this sugar is always weak and may not appear before forty-eight hours.

Although there is as a rule a sharp distinction between *S. pullorum* and *S. gallinarum* in the fermentation of maltose, dextrin and dulcitol, there may appear slight positive reactions with *S. pullorum* in maltose or dextrin. This is due to the fact that maltose is easily broken down in sterilization and some lots of dextrin contain sufficient impurities to permit some fermentation. Sterilization in the autoclave at 10 pounds produced less hydrolysis in maltose than sterilization in the Arnold. Dulcitol always gave a sharp distinction between the two species.

While more than half of the *S. pullorum* strains regularly produced gas from all of the carbohydrates fermented, there were quite a few strains that failed to produce gas from some carbohydrates and only occasionally produced gas from others. These irregular strains include most of Hadley's (Hadley, Elkins and Caldwell, 1918) anaerogenic strains, but culture 162 which was one of Hadley's typical strains of *Bact. pullorum B* regularly produces gas.

The results of these tests do not show any difference between cultures from diseased adult fowls and from chicks or the ovaries of apparently healthy adult fowls. Most of the more recently isolated cultures appear among the irregular strains, while only a few of the older cultures appear there. This indicates that gas production from ordinary carbohydrate media tends to increase

TABLE 2

Fermentation of carbohydrates

	ARABINOSE		XYLOSE		RHAMNOSE		GLUCOSE		LEVULOSE		MANNOSE		GALACTOSE		MALTOSE	LACTOSE	SUCROSE	RAFFINOSE	DEXTRIN	INULIN	DULCITOL	MANNITOL		ERYTHRITOL	SALICIN
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Gas	Acid	Acid
S. pullorum: 12–18, 20, 34, 99, 123–129, 162, 167, 170, 220, 223–230, 257, 258, 272, 277	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 19	+	−	+	+	+	+	+	− (+)	+	−	+	− (+)	+	− (+)	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 102A	+	+	+	+	+	+	+	− (+)	+	−	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 102B	+	+	+	+	+	+	+	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 117	+	−	+	−	+	−	+	− (+)	+	− (+)	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 118	+	−	+	−	+	−	+	− (+)	+	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 122	+	−	+	−	+	−	+	− (+)	+	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 165	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 168	+	+	+	+ (−)	+	+	+	− (+)	+	+	+	− (+)	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 169	+	+	+	+	+	+	+	− (+)	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 224A	+	+	+	+	+	−	+	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−

S. pullorum: 233	+	−	+	− (+)	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 234	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 244	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 245	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 246	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 247	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 271	+	−	+	−	+	−	+	+	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 273	+	−	+	−	+	−	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−
S. pullorum: 275	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−
S. pullorum: 276	+	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. gallinarum: 38, 110, 115, 116, 147, 148, 158, 161, 163, 164, 202, 206–216, 221, 235–237	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	+	−	−	−
S. gallinarum: 45	+	−	+ (−)	−	+	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	+	−	−	−
S. gallinarum: 47, 66	+	−	−	−	+	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	+	−	−	−
S. gallinarum: 65, 88	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	+	−	−	−

Note: Figures in parentheses indicate exceptional findings.

with the length of time a culture has been kept on artificial media. This is, however, not a universal rule, as some strains that have formerly produced gas regularly, now fail to produce gas from some carbohydrates. Since every laboratory obtains different results in regard to gas formation by *S. pullorum* and since different workers in the same laboratory or the same workers at different times do not always get the same results it is probable that slight variations in the media produce relatively large differences in gas formation. The authors are taking up a special study of this question and hope to be able to make a report in the near future.

ANTIGENIC STUDY

For the inoculation of rabbits, cultures were selected that represented all of the various peculiarities that were known at the time. From *S. pullorum* the following cultures were selected: An early and a later strain from Dr. Rettger (Nos. 20 and 122); an early and a later strain from Dr. Gage (Nos. 12 and 128); two strains which Hadley had determined to be *S. pullorum B* (Nos. 118 and 162); a typical and an irregular strain isolated very recently in this laboratory (Nos. 224 and 234 respectively). The selections to represent *S. gallinarum* were Klein's original (No. 88), Moore's original *Bact. sanguinarium* (No. 115); a strain from the Pasteur Institute (No. 206), and a recent isolation by this laboratory (No. 235). For the sake of comparison a strain of the human typhoid bacillus was also used.

These organisms were grown on meat infusion agar slants for twenty-four hours, the growth washed from the slants with the aid of sterile saline and the suspensions heated in a water bath for thirty minutes at 60°C. The suspension was standardized by the Gates (1920) method at a density between 1 and 2 cm. and was then diluted so that the final density was approximately a computed 8 cm.

Young, healthy male rabbits were given five intravenous inoculations of killed bacterial suspension at intervals of two to three days. The amounts used in these injections were: 0.25 cc., 0.5 cc., 1.0 cc., 1.5 cc., and 2.0 cc., respectively. Test bleeding

was made on the fifth day after the last inoculation. In every instance a favorable titer was obtained and the final bleeding was on the seventh to ninth day after the last injection.

In preparing the bacterial suspensions for the long series of titrations, the organisms were grown in large plates on meat infusion agar. The growth was washed off with 0.8 per cent sodium chloride solution containing 0.5 per cent phenol, and the suspension was diluted to an appropriate density with the same reagent. This density corresponded to approximately 3 cm. by the Gates method.

The dilutions of the antiserum were such that each succeeding tube had twice the dilution of the one just preceding, e. g., 1–40, 1–80, 1–160, etc. Antigen and antiserum were then mixed in equal parts, giving a final antigen dilution of 6 cm. and a final serum dilution of 1–80, 1–160, 1–320, etc. Each antiserum was titrated against each antigen and controls were uniformly run. The tubes were incubated at 37°C. for eighteen hours and this was followed by a few hours in the ice box. Tubes were read out for agglutination with a lens. More than twenty-five hundred titrations were made in this series. All antigens were titrated against the same serum at the same time, in fact the serum was diluted in flasks and pipetted out to the agglutination tubes so that all antigens would be mixed with serum of exactly the same strength.

The results have been summarized in table 3. Only the denominator of the fraction representing the limit of agglutination between a given antigen and serum has been given. The regular *S. pullorum* strains are listed first followed by those giving some irregular fermentations. The division of the strains of *S. gallinarum* is a natural one based on the agglutinative properties of the various antigens. Certain strains which were agglutinated to the same degree by the same antisera are placed above, followed by the less regularly agglutinated strains.

A study of table 3 seems to show that there are no conspicuous antigenic differences within the groups studied. At the same time there are some variations which are hard to explain.

In the titration of pullorum antigens against pullorum antisera

TABLE 3

*Agglutination limits**

NUMBER	S PULLORUM								S. GALLINARUM				
	12	20	122	224	128	234	118	162	206	88	115	235	77†
S. pullorum													
12	20480	20480	40960	20480	10240	10240	40960	5120	2560	5120	10240	10240	1280
13	20480	10240	20480	10240	10240	10240	20480	5120	10240	5120	10240	5120	1280
14	10240	10240	10240	10240	2560	2560	10240	5120	2560	1280	5120	0**	640
15	10240	10240	10240	10240	5120	10240	20480	5120	2560	10240	5120	5120	1280
17	10240	5120	20480	5120	5120	10240	20480	5120	2560	2560	5120	10240	640
18	20480	10240	20480	20480	10240	10240	20480	5120	5120	5120	5120	5120	640
20	20480	20480	40960	20480	10240	20480	40960	10240	10240	5120	10240	10240	2560
34	20480	20480	20480	20480	10240	10240	40960	5120	5120	5120	10240	2560	2560
99	10240	20480	10240	10240	10240	10240	20480	10240	0	2560	5120	5120	1280
123	20480	20480	40960	10240	10240	10240	20480	5120	2560	5120	5120	10240	640
124	10240	5120	5120	5120	1280	1280	2560	2560	2560	1280	1280	5120	320
125	10240	5120	10240	2560	5120	2560	10240	2560	1280	2560	5120	5120	320
126	10240	10240	20480	10240	5120	10240	40960	5120	10240	2560	2560	5120	640
127	10240	5120	10240	10240	5120	5120	20480	5120	5120	2560	2560	5120	640
128	10240	10240	20480	10240	10240	10240	40960	5120	5120	2560	5120	5120	640
129	5120	2560	5120	2560	1280	2560	5120	1280	1280	640	5120	2560	160
162	5120	10240	10240	10240	2560	10240	20480	2560	2560	2560	5120	5120	640
167	10240	10240	20480	10240	5120	5120	20480	5120	2560	5120	2560	2560	640
170	10240	10240	20480	20480	5120	10240	40960	5120	5120	5120	5120	5120	1280
220	10240	10240	20480	10240	5120	10240	20480	10240	2560	5120	5120	5120	640
223	10240	10240	10240	10240	5120	5120	20480	10240	5120	5120	2560	5120	640
224	10240	10240	20480	20480	10240	20480	40960	10240	5120	10240	10240	5120	1280

225	10240	10240	10240	10240	10240	10240	40960	10240	5120	5120	10240	5120	1280
226	10240	5120	10240	5120	1280	2560	10240	1280	2560	2560	5120	1280	320
227	10240	10240	20480	10240	5120	5120	40960	5120	5120	2560	5120	5120	640
228	10240	20480	20480	20480	10240	10240	40960	5120	2560	2560	5120	5120	1280
229	10240	10240	10240	20480	10240	20480	40960	10240	5120	5120	10240	5120	1280
230	5120	5120	5120	5120	1280	10240	20480	2560	5120	5120	5120	5120	160
257	10240	5120	20480	10240	5120	10240	20480	5120	2560	2560	5120	5120	640
258	20480	10240	10240	10240	5120	10240	20480	5120	10240	5120	10240	10240	1280
272	10240	5120	5120	5120	2560	10240	20480	5120	2560	2560	2560	5120	320
277	20480	20480	40960	5120	20480	10240	20480	10240	5120	5120	5120	5120	2560
19	10240	10240	10240	2560	5120	10240	20480	2560	5120	5120	1280	5120	640
102A	20480	10240	20480	5120	10240	10240	20480	5120	1280	0	0	10240	1280
102B	20480	10240	20480	10240	10240	20480	40960	10240	10240	10240	10240	10240	2560
117	20480	10240	20480	10240	10240	10240	20480	5120	5120	2560	2560	5120	1280
118	10240	10240	20480	5120	10240	2560	20480	5120	1280	1280	2560	5120	160
122	20480	20480	40960	20480	10240	10240	40960	5120	10240	2560	10240	10240	1280
165	5120	20480	10240	2560	2560	2560	20480	1280	2560	2560	1280	1280	0
168	20480	20480	10240	5120	2560	2560	20480	2560	2560	2560	2560	5120	320
169	10240	10240	20480	10240	5120	10240	20480	5120	2560	1280	2560	5120	640
224A	10240	10240	20480	10240	10240	10240	40960	5120	10240	10240	5120	10240	2560
233	20480	10240	40960	5120	10240	20480	40960	10240	10240	10240	5120	10240	2560
234	20480	20480	40960	5120	10240	20480	40960	10240	10240	10240	2560	5120	2560
244	20480	5120	10240	10240	10240	10240	20480	10240	5120	10240	5120	2560	1280
245	20480	20480	40960	20480	10240	20480	20480	10240	5120	10240	5120	10240	1280
246	20480	20480	40960	20480	10240	20480	40960	10240	5120	10240	10240	10240	2560
247	10240	10240	10240	10240	5120	10240	20480	10240	5120	2560	5120	5120	1280
271	10240	5120	10240	10240	10240	20480	20480	5120	5120	2560	5120	5120	1280
273	20480	10240	10240	5120	2560	5120	20480	2560	2560	2560	10240	10240	320
275	20480	20480	20480	10240	5120	10240	20480	5120	5120	5120	5120	10240	640
276	20480	20480	40960	20480	10240	20480	20480	10240	10240	5120	10240	10240	1280

TABLE 3—*Concluded*

NUMBER	S. PULLORUM								S. GALLINARUM				
	12	20	122	224	123	234	118	162	206	88	115	235	77†
						S. gallinarum							
38	5120	2560	10240	5120	1280	5120	10240	2560	1280	2560	5120	2560	320
110	2560	2560	2560	2560	640	1280	2560	640	1280	1280	2560	640	160
115	5120	2560	10240	640	2560	2560	10240	1280	2560	1280	640	2560	2560
116	2560	1280	5120	2560	1280	2560	5120	1280	1280	5120	2560	1280	0
147	2560	2560	2560	2560	1280	5120	10240	1280	5120	5120	5120	2560	320
148	10240	10240	10240	5120	2560	2560	10240	2560	5120	5120	5120	2560	320
158	1280	2560	2560	2560	640	2560	10240	1280	2560	1280	5120	2560	160
161	1280	2560	5120	2560	2560	2560	5120	1280	1280	1280	5120	2560	160
163	2560	1280	2560	2560	640	1280	10240	640	1280	1280	2560	2560	0
164	5120	5120	5120	5120	2560	5120	10240	2560	2560	2560	2560	2560	320
202	10240	5120	10240	640	2560	5120	10240	1280	5120	5120	1280	2560	320
206	10240	20480	20480	10240	5120	10240	10240	5120	10240	5120	10240	5120	320
207	10240	10240	20480	5120	2560	5120	10240	2560	5120	10240	5120	2560	160
208	10240	10240	20480	5120	2560	5120	10240	5120	5120	1280	2560	2560	160
209	2560	5120	1280	2560	2560	2560	10240	1280	2560	2560	1280	2560	0
210	5120	10240	20480	2560	2560	2560	10240	2560	2560	1280	5120	2560	160
211	10240	10240	20480	5120	5120	10240	10240	2560	2560	2560	10240	2560	640
212	10240	10240	20480	2560	2560	5120	10240	2560	5120	2560	5120	2560	160
213	2560	2560	2560	2560	5120	5120	10240	2560	5120	2560	10240	2560	320
214	5120	10240	10240	2560	5120	2560	5120	1280	2560	1280	10240	2560	160
215	5120	10240	10240	2560	1280	2560	10240	2560	2560	2560	5120	5120	160
216	10240	10240	20480	5120	5120	5120	20480	2560	5120	2560	5120	5120	320
221	10240	10240	20480	2560	2560	5120	10240	2560	2560	5120	2560	2560	160

235	5120	20480	10240	2560	2560	2560	10240	2560	2560	1280	2560	2560	320
236	10240	10240	20480	2560	1280	2560	20480	5120	2560	1280	2560	5120	320
237	5120	5120	10240	2560	1280	2560	10240	1280	1280	5120	5120	10240	80
45	1280	80	2560	640	2560	1280	640	160	320	5120	5120	1280	0
47	1280	1280	320	640	320	1280	1280	640	160	5120	640	640	0
65	1280	640	5120	1280	1280	1280	5120	640	2560	1280	1280	640	0
66	640	1280	1280	640	640	1280	5120	640			640		0
88	1280	640	640	160	320	320	640	160	320	10240	320	640	0
77†	0	0	0	0	0	0	0	0	0	0	0	0	5120

* Antigens are shown at the left and antisera at the top.

† *Eberthella typhosa* (Zopf), Rawlings type (from Mulford laboratories)

** The term "0" indicates no agglutination in 1–80.

the uniformity in the degree of agglutination is very striking. In any one serum no antigen agglutinates more than eight times as well as any other antigen. Furthermore, the weakly agglutinating antigens give a rather uniformly low titer with all sera tested, while the more readily agglutinating antigens give a relatively high titer with all sera. No indication of grouping is therefore apparent.

In the titration of pullorum antigens against gallinarum antisera the degree of agglutination was uniformly somewhat lower and variations were somewhat greater. There has appeared nothing unusual, however, except for occasional serum-antigen combinations that failed to give agglutination even in the lowest dilution of the serum (1–80). These sporadic cases can in no way be correlated with antigenic groups and must for the present remain unexplained.

In the titration of gallinarum antigens against pullorum antisera the degree of variation is still more pronounced. The division, however, is again into weakly agglutinating antigens and more strongly agglutinating antigens. For the sake of comparison the antigens showing the lowest degree of agglutination with pullorum sera have been listed at the lower end of the table (cultures 45, 47, 65, 66 and 88).

The degree of agglutination between gallinarium antigens and gallinarum antisera is very nearly the same as in case of gallinarum antigens and pullorum antisera. The same group of cultures is again more or less set off from the rest as showing the lowest degree of agglutination.

In the *Eberthella typhosa* antiserum both pullorum and gallinarum antigens agglutinated only to a moderate degree, the pullorum antigens agglutinating somewhat better than the gallinarum antigens. In fact, some of the gallinarum antigens failed to agglutinate in this serum even in the highest concentration used. Among these antigens is the entire group at the end of the list. The typhosa antigen failed in all cases to agglutinate in the pullorum and gallinarum antisera.

Outside of the five gallinarum strains mentioned, there is nowhere any indication of antigenic groups. These five strains

may possibly be considered as forming an antigenic group feebly set off from the rest. They fail to agglutinate well with pullorum and gallinarum antisera and do agglutinate as a rule fairly readily with the antiserum of one of their own number, strain 88. The division, however, is not at all clearly marked as strain 65 does not agglutinate strongly with antiserum from strain 88 and other cultures such as 110, 116, 147, 161 and 163 show only slightly stronger agglutination in other sera than do the members of this group.

GENERAL DISCUSSION

One of the more important questions in connection with these two organisms is their position in the system of classification. The typical aerogenic *S. pullorum* has long been accepted as a member of the genus *Salmonella* but the anaerogenic form is rather a stumbling block for those who use gas production as the essential criterion. Depending upon the classifier, *S. gallinarum* is likely to be found either in the genus Eberthella or the genus Salmonella.

One thing has definitely been shown, and that is that *S. gallinarum* bears an extremely close relationship with *S. pullorum.* Any classification that seeks to throw the two organisms into widely separated groups is only defeating the purpose of the system.

It must be accepted too, that there is a certain antigenic similarity between these two organisms and *Eberthella typhosa,* but it has been shown in this paper that this relationship is not as remarkable as certain workers have been led to believe.

Although the members of the old colon-typhoid group are becoming more and more distinct and defined, there still exists a great deal of confusion and all of the systems of separating this large group into sub-groups or distinct genera must for the present still resort to some very artificial barriers. One of the more obvious lines of division has been the presence or absence of gas formation in fermentable sugars. This has recently been used very much as the presence or absence of motility was formerly used. It appears, however, that in the case of the two species

considered the distinction on the basis of the presence or absence of gas formation is entirely too artificial, separating into two genera organisms that otherwise scarcely show specific distinctions. To avoid this unnatural division it seems necessary to regard the two species as one unit and place them in the genus to which they collectively show the greatest relationship. This beyond a doubt is the genus Salmonella in which Lignières (1925) originally placed his organisms of fowl typhoid and in which both organisms would fall according to the grouping by Krumwiede and Kohn (1917) and by Park, Williams and Krumwiede (1924). The grouping of these authors ignores gas production and excludes from the genus Salmonella organisms which do not ferment rhamnose and which do ferment lactose, sucrose or salicin.

With this in view the following definitions are advanced for these organisms.

Salmonella pullorum (Rettger) (1909).

Gram-negative, non-spore-forming, non-motile rods. Growth on solid media resembling *Esch. coli*, but much less vigorous. Acid is produced in media containing arabinose, xylose, rhamnose, glucose, levulose, galactose, mannose and mannitol. Gas may or may not be produced. Does not ferment lactose, sucrose, raffinose, maltose, dextrin, dulcitol, inulin, erythritol or salicin. Does not produce indol or liquefy gelatin. Usually produces hydrogen sulphide. Indistinguishable antigenically from *Salmonella gallinarum* (Klein). Regarded as the causative agent of bacillary white diarrhea of young chicks but found in adult birds in localized infections or as producing a disease that can not be distinguished from fowl typhoid.

Salmonella gallinarum (Klein) (1889).

Gram-negative, non-spore-forming, non-motile rods. Growth on solid media resembles that of *Esch. coli*, is less vigorous, but is more vigorous than that of *S. pullorum*. Never produces gas in any carbohydrate media. Acid is produced in media containing arabinose, rhamnose, glucose, levulose, galactose, mannose, maltose, dextrin, dulcitol and mannitol. Does not ferment lactose, sucrose, raffinose, inulin, erythritol, or salicin. Does

not produce indol or liquefy gelatin. Usually produces hydrogen sulphide. Most strains are indistinguishable antigenically from *S. pullorum*. Regarded as the causative agent of fowl typhoid but possibly also associated with diarrhea-like disturbances in chicks.

For routine identification purposes the following scheme is in use in this laboratory.

Gram-negative, non-spore-bearing rods from avian sources are considered. These are inoculated into media containing glucose, lactose, sucrose, and dulcitol respectively. If the glucose is fermented with or without gas, but none of the others are fermented the organism is *S. pullorum*. If both glucose and dulcitol are fermented but not lactose or sucrose the organism is *S. gallinarum*. The use of the glucose media is necessary to place the organism in the group. The lactose serves to eliminate coli-like organisms, the sucrose eliminates cholera-like bacteria, while the dulcitol serves to distinguish between *S. pullorum* and *S. gallinarum*. Maltose or dextrin might be substituted for dulcitol but the latter has been found much easier to prepare and to give very reliable results. To reduce cost dulcitol is used in 0.5 per cent solution in place of 1 per cent.

The results of this investigation do not entirely clear up the difference in results obtained with various antigens in the routine test for pullorum reactors. The fact that some antigens will agglutinate in a given serum as much as 8 times as well as others explains some of the results, such as the fact that some antigens will usually detect more reactors than others; but the fact that the weaker agglutinating antigens will in certain flocks detect more reactors than the more readily agglutinated strains still remains unexplained.

SUMMARY

A study of the carbohydrate fermentation of 52 strains of *S. pullorum* and 31 strains of *S. gallinarum* shows that the members of each group agree very closely in the production of acid from these substances. *S. pullorum* usually produces gas from fermentable carbohydrates while *S. gallinarum* never produces gas.

The titration of antigens from these strains against antisera

from 8 representative strains of *S. pullorum* and 5 representative strains of *S. gallinarum* as well as one strain of *Eberthella typhosa* reveals no definite antigenic differences between the two groups of avian pathogens nor does it reveal any sub-groups with the exception of a slight differentiation within *S. gallinarum*.

Both pullorum and gallinarum are regarded as belonging to the genus Salmonella.

A method for the rapid identification of the two species consists in passing suspected avian pathogens (gram-negative rods) through glucose, dulcitol, lactose and sucrose.

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[2] The recent literature of this subject is reviewed in Bulletin 204 of the R. I. Agr. Exp. Sta.

PROBLEMS OF THE BACTERIOLOGIST IN HIS RELATIONS TO MEDICINE AND THE PUBLIC HEALTH[1]

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When one is confronted with the rare—shall I say—opportunity of writing a presidential address, the temptation is great to try one's wings at oratory. However, while bacteriologists, occasionally born, can sometimes be made, orators are always born. It seemed better, therefore, in seeking honorable solution for the task before me to suppress the impulse to deliver an oration and instead of this to talk; and, moreover, to talk as simply as I can about matters that have long been in my mind, instead of painfully seeking for a subject for the kind of presidential address which my conscience tells me I ought to deliver. The subjects that I have chosen for my discourse are therefore to some extent disconnected, but in my desire to say things that have seemed to me important I have ventured to sacrifice form to content. Many of the opinions that I am about to express, especially in the third section in which I deal with the patenting of scientific discoveries, are sure to provoke adverse criticism. However, they represent seriously weighed opinions which I have felt are entitled to consideration by a group like the one represented in this Society. If I arouse opposition in many of you and approval in a few of you, I shall at least know that my breath in passing the vocal cords has gathered words that will reach you as something more than forcible expiration.

I. ON A GROWING MISUNDERSTANDING BETWEEN THE MEDICAL PROFESSION AND THE SCHOOLS IN REGARD TO THE TEACHING OF THE SCIENCES

Every progressive movement creates its own reactionary response. This is wholesome and keeps the wheels of progress

[1] Presidential address delivered before the Society of American Bacteriologists, Philadelphia, Pa., December 29, 1926.

in the middle of the road. As a rule, however, in most human affairs the reactionary party is that of the old and skeptical who are applying the brakes because they fear a disturbance of those conditions which have given them their own successes and privileges. When this is so it is normal and a well recognized phenomenon which can be left for solution to time and mortality. In medical education at the present time, unfortunately, this general rule does not hold. It would be convenient and reassuring to dismiss the criticism and objections that are made in regard to the conduct of our medical schools as the grumblings of old fogeys. But this would neither be true to the facts nor would it serve to clarify a misunderstanding which is not without grave dangers to the healthy development of medical progress. For it is well to remember that however firmly established these medical schools may be in their university connections, they should—indeed must—have the sympathy and support of the profession at large. For the progress of medicine will continue to be so rapid that—to serve the public and uphold standards—there must be maintained a continuity of coöperation between the educational products of successive decades by which the developments of knowledge may be constantly fed, in the form of the younger graduates, to the central stream of medical activity, where ripened clinical experience will apply them to the problems of practice.

There is threatening to develop a gap in this continuity at the present time. The bitter critics of the medical schools are not the old, the intellectually lame and the halt. The most serious protests against the policy of extensive—and therefore prolonged and expensive—scientific training are coming from active practitioners, some of the most successful in their respective fields and, therefore, eminently entitled to notice. If we exclude all the trivial, thoughtless and facetious disapproval which every rapidly progressive movement must encounter, there still remains a formidable and growing protest of dissenting voices from substantial sources. These are being expressed in important lay and medical publications and in addresses before medical societies, to say nothing of innumerable conversational comments

wherever practicing physicians and medical teachers meet for discussion. The series on "Education and Service" published in the Journal of the American Medical Association by Dr. Pusey, the presidential address to this Association by Dr. Wendell Phillips are fair examples of the importance of the criticisms to which we refer. They are all of them earnestly intended, thoughtfully expressed and based upon apprehension caused by a number of obvious failures of the present system to meet requirements.

The basis of such criticisms has been most clearly presented by Pusey, whose articles are admirable in flatly formulating the issues. "We are producing only a very costly sort of physician," he states, "and are not now producing men to do the ordinary service of medicine for ordinary people." He reiterates the well-known facts that modern graduates will not go to the country, that the family physician is disappearing and leaving an open field for the quack, the midwife and the faddist. These and many other evils he lays at the door of the medical schools. Students are crammed full of useless facts, he asserts, and if teaching methods were not quite as inefficient as he believes them to be, entrance requirements could be curtailed, and the courses shortened to three years without loss, possibly gain, in the quality of the product. He accuses the schools of emphasizing research at the expense of teaching, and states that "research and education are not the same things." We might with profit quote much more extensively from Dr. Pusey's article, for he voices clearly and unashamed the views of those who hold faulty educational methods almost solely responsible for present failures in medical service. For our present purposes we must overlook the obvious contributory importance of other factors such as economic pressure, social readjustments and the growing necessity of equipment, coöperation and specialized advice for the adequate modern medical service to which even "ordinary people" are entitled, and may confine ourselves entirely to a consideration of the alleged errors in methods of instruction.

Those who are responsible for the conduct of the schools are fully aware of the defects which he points out. The ultimate purposes of adequate medical service for which Dr. Pusey strives

are also those of the teaching institutions. The difficulties to which he gives too little consideration are those created by the prodigious volume of scientific knowledge contributed to the field of medicine in a relatively short time; and it would seem obvious that increased requirements for knowledge and technical ability can not be satisfied by shorter periods of training and neglect of instruction in fundamental principles.

Let us consider for a moment what eminent clinicians expect of the modern graduate in medicine. We repeat a quotation used by Dr. Wendell C. Phillips from an article by Dr. Billings: "The ideal general practitioner 'must have a good knowledge of general and physiologic chemistry, of human anatomy and physiology, of pharmacology, of general pathology,—with constant training, through the four years of residence, in morbid anatomy,—of epidemiology, immunology and psychology. In the clinical branches he must have the training of his brain, special senses and hands in the recognition of disease. He must learn to use the simple diagnostic aids in the form of instruments and laboratory tests. With this knowledge of the methods of diagnosis, he must have constant and daily opportunity to study disease as expressed in the ambulatory and ward patients. He must have an opportunity to observe the etiologic relations of occupations, environment, social conditions and other factors to disease. He must learn and recognize the importance of community as well as individual disease' . . . ," etc. "Superman" is the thought that comes to the mind on reading this. Of course it cannot be accomplished—but if it can be approached at all, it is quite clear that the curtailed curriculum is not a promising method.

It may be admitted that, with constantly growing accumulations of fact and too slow a transformation of older methods, there has been a period of over-teaching which is still to some extent upon us. But in facing a task difficult beyond precedent in the educational field, experienced medical teachers are aiming, not at the immediate and temporary solution of the present transitional difficulties, but at an eventual provision of men capable of—as Stockard puts it—"understanding and applying scientific biologic knowledge."

Medicine is not a trade that can be drilled into an unprepared mind by shop methods. While it is not entirely a science, there is much reason for believing that a thorough comprehension of the chemical and biological principles underlying the pathology and therapy of the diseases met in daily practice are absolutely indispensable to the later acquisition of the equally important technique or art of practice. Is it conceivable that even to-day a practicing physician can intelligently apply modern therapeutic methods to cases of diabetes, nephritis or any or the other metabolic diseases without at least a fundamental training in organic and biologic chemistry? Is it desirable that he employ the various agencies of specific diagnosis and therapy like parlor tricks or patent medicines, without understanding the principles by which their use is rendered logical? Moreover, medicine is moving forward, and the student of today will be in practice twenty and thirty years from now. Is there the slightest hope that he may be able to follow in the footsteps of advancing knowledge unless a considerable part of the short years of learning is given to the fundamental soil from which this new knowledge will grow? What hope would there be for our chemical industry, if students were apprenticed in factories without knowledge or experience of physical or chemical reactions? What of the future of our engineers, if they were taught with formulated tabulations instead of drilled mathematics? The scaffoldings of medicine are and will be constructed from biological, chemical and physical principles. If these are reasonably understood, available knowledge can be utilized; and the progress of the future can be mastered. Without such training the graduate is left suspended in midair instead of standing on a ladder which he can climb. He is like a musician who has learned a few pieces without reading notes; and the difference between him and an intelligent midwife ten years hence will be not much more than that between Dr. Slop and Susannah, who assisted at the birth of Tristram Shandy. Dr. Pusey says: "The resourceful man has his equipment under his hat and in his hand, and he gets it, not in the laboratory, but in clinical experience." We may grant a considerable hat, but not even the hat of the late Houdini—

while it might be made to disgorge amboceptor rabbits and guinea pigs—can be relied upon for Wassermann equipments, electro-cardiograms, potentiometers or metabolism laboratories.

It is true that methods of teaching have become experimental rather than didactic, but this is in the interests of stimulating the students to draw conclusions rather than feeding them on dogmatic statements. That errors are made and pedagogic principles transgressed, no one will deny—but the effort is there; and the suspicion of attempts to make investigators of students is quite obviously refuted by the intrinsic impossibility of such an endeavor.

Let it be granted that the graduate from a medical school to-day is not a finished practitioner. Let us even concede that he is not as capable of taking charge in the sickroom on the day of graduation as his predecessors of the last generation. All this is true, but the fault lies in the growth of medicine; the remedy lies, not in the schools, but in the hospital years and in the tutelage of his elders in the field. The situation cannot be met by aiming our educational methods at the rapid production of "cheap doctors for the country"—a sort of "Muzhik" doctor, or "Feldscher"—justified in a democratic country only by the possibility that the farmers are a tougher breed, but otherwise untenable.

We would suggest that the critics of the medical schools have patience with those attempting to perform a difficult service; that they come to the schools as observers; that they attend clinics by some of their full-time colleagues and examine whether there is not something here, different from that which is given later by the practicing clinicians, and not more valuable perhaps, but equally essential to the full development of a modern physician. For the physician of the future will be a different person from the physician of the past. He will be no finer than the Oslers, Delafields, Jacobis, James', Shattucks and others who inspired our youthful ambitions. But to be equally fine and capable of meeting the responsibilities which accumulating knowledge is laying upon their shoulders, he must needs be differently trained. For in medicine, more than in other professions, ignorance—usually a negative failing—becomes a positive one; and a phy-

sician who neglects, through lack of knowledge, to apply available methods of interference may be as morally guilty as though he had hit his patient on the head with a hammer.

As to the eventual adequate supply of men willing to submit to this rigid discipline for relatively modest material futures—may we not safely trust to that consoling human folly that has made Christianity a reasonably successful institution?

II. ON THE INTERDEPENDENCE OF RESEARCH AND TEACHING

A considerable part of the criticism aimed at the modern medical school boils down to the charge that too much emphasis is placed upon research, and too little attention given to the teaching of existing knowledge. Defense against this accusation involves a general discussion of the functions of the departments which instruct in the preclinical sciences, and calls for some consideration of the assertion—frequently coupled with such criticism—that "research and education are not the same things" and had better be separated.[2] This last suggestion, we believe, is a pernicious one chiefly because it fails to recognize the importance of a high order of scientific background for that most difficult of all teaching, the exposition of the simple, basic facts to beginners—a task that is incompletely performed unless the information conveyed arouses curiosity and awakens the critical faculties.

If the scientific laboratories of medical schools were to be planned to serve purely teaching functions, they could be organized cheaply and simply; but, though it sounds a paradox, such limitations would defeat their very teaching purposes. We are admitting, of course, that medical students cannot be graduated as investigators, and it is surprising that anyone should assume that otherewise reasonable educators are not aware of this. But we assert that the separation of investigative activities from departments engaged in the exposition of any subject in medicine would irreparably damage their purely educational effectiveness.

[2] See Dr. Pusey's article in the Journal of the American Medical Association for May 15, 1926; also, J. B. S. Haldane in the Atlantic Monthly for February, 1926. Similar views have been expressed by many other writers—chiefly clinicians.

Men who devote themselves exclusively to the teaching of subjects as actively advancing as are the various departments of medical knowledge, with little or no interest in investigation, show themselves by this testimony inadequately endowed with intelligent curiosity and imagination. They are manifestly pedants whose faculties are left unstirred by the most fascinating invitation to intellectual adventure that modern thought has to offer. The teacher of a growing science who abstains from inquiry is as much out of place in a laboratory as a prohibitionist in a wine cellar. Moreover, to teach adequately and inspiringly signifies not the dispensing of other men's thoughts but, rather, the distillation of accumulated knowledge through the mind of the expositor, carrying over a part of himself in the process.

Over-emphasis on mere pedagogy in the teaching of a science—a real danger, if recent critics are given their heads—may easily do to medicine what it has done, in our own experience, to science courses in some of our teachers colleges. In at least one institution known to us the University chemical department did not, a few years ago, accept as equivalents courses given on the same subjects to students in its own teachers college. These things are not cited in order to belittle the desirability of developing better methods of instruction in the sciences. They are mentioned in support of the view that the best and most inspiring teachers—those whose spirits have catalyzed the latent capacities of their students, and have transmitted to them something more than forgettable facts, are the investigators whose teachings have been interwoven with that joy of their work that is born of the spirit of exploration. We may paraphrase the remark attributed to Sir Thomas Lipton that "married men make the worst husbands" with the truism that the professional pedagogue makes a poor teacher of science. He will teach—by a perfect technique, perhaps—the things he has read in a printed book, or the things that he has been told and shown, but his teachings will be as uninspiring to his students as Tomlinson's sins were to the devil in Kipling's poem.

Incidentally, in spite of the encouragingly generous recognition by the universities of the truth of these principles, it is

growing annually more difficult for the scientific laboratories generally to recruit the most promising young men for their important tasks. Competitive opportunities in the clinics, in research institutes and in the industries, with better pay and less routine, are luring many of them away. And in this alone there is a sufficient element of danger, without added discouragement from those who would reduce these teaching departments to the status of the Mohammedan schools where the scholars study the philosphy of the Koran by chanting it in unison. It might mean that, gradually, intellectual leadership in fundamental investigation would drift away from the universities—and if this should go far enough, these institutions, upon which, after all, the intellectual development of the future depends, would cease to be truly universities and become mere teaching high schools, without inspiration and without hope.

Let us admit that there are rare and precious spirits with a genius for investigation who have no talents or desires for teaching and who, in the interests of husbanding our gifts, should be protected from the interruptions of the class-room. It is an obvious waste for men of the Loeb and Theobald Smith type to be burdened by routine, be it ever so limited. These, however, are a small minority for whom exceptional provision is justified and—as a matter of fact—provided. But, fortunately, men with excellent teaching ability have often been capable of considerable originality in research; and many others with investigative talents of no mean order have, not exceptionally, possessed sufficient ability to expound; and any defects in pedagogic technique have been amply compensated for by the enthusiasm they have conveyed to their students. We need go no farther back than the present generation to illustrate the contention that the teachers, those who have most influenced medical students and whose spirit is most alive among the younger practitioners of our country to-day, were men actively and productively engaged in investigation. Would a system of separation of these two functions have developed Welch, or Remsen, or Edmund B. Wilson, or Chittenden, or Halstead, or Henderson, or Huntington,

or Lusk, or Abel, or Folin, or a host of others whose pupils are shaping the destinies of American medicine today?

It is too often assumed that originality and scholarship are incompatible with common sense. But the professor and scientist who holds an egg in his hand while he times the boiling of his watch, or who looks under the dresser for a collar button that is in his shirt, is more frequent in the humorous weeklies than in real life. It is quite safe to have confidence that those who are studying the problem as a whole realize that medical students must be given primarily the information which will prepare them for the duties of their profession. But they believe that such instruction cannot safely be limited to a recital of facts. They believe—or, at any rate, a great many of them do—that science and the student are best served in establishments in which every student may have opportunities for contact with men engaged in active investigation; and in which no instructor shall be entirely removed from the stimulating association with the eager and intelligent young minds that demand reason and proof as well as dogma from their teachers. Scope and the selection of material, and not the limitation of intelligent curiosity, are the real problems; and though, undoubtedly, many mistakes are being made and the pendulum has perhaps often swung too far toward irrelevant details—these are natural reactions against the dull lecture systems of other days.

The problem—and we do not claim that it has been solved—lies perhaps in a closer coördination of the laboratory teaching with its clinical applications. But whatever the solution may be —and it is being earnestly sought—it surely will not be found in a sterilization of the teaching force, which indeed more often needs a Voronoff than a general surgeon.

Let us be thankful that the medical schools are so closely allied to universities, so strongly influenced by the fundamental departments of physics, chemistry and biology, and that university authorities are recognizing the important principles that true education signifies the planting of seeds in fertile soil, and not the feeding of the ripened fruit of the past season.

III. ON THE PRACTICE OF PATENTING DISCOVERIES IN THE FIELD OF MEDICINE

We are living in a time in which the principles of business and trading have become more than ever before the arbiters of personal and national standards. This, for many reasons that would carry us far beyond our present purposes, is a logical consequence of historical and economic developments. At all times, however, the pendulum of contemporary tendencies swings far beyond the necessary point into levels at which matters are involved that are not subject to time and circumstance; which are based upon the natural laws of conscience and professional obligation, matters that are not altered by changed political and economic conditions. This, perhaps, is the difference between a trade and a profession.

One cannot practice a profession like a trade, and this is not idealistic moonshine, it is practical common sense in the interests of achievement and success. The progress of the past in the sciences as in the arts has been, and will be in the future, determined by impulses and ambitions that would suffer were they swept into the currents of business development. And the methods and procedures, right, necessary and inevitable in commercial organization, would prove serious detriments and impediments to the purposes of sciences like our own, unless applied with cautious limitation to the necessary minimum.

I am referring to a matter which I have long desired to bring to the attention of the Society of Bacteriologists, and which has been much discussed during the last few years without being crystallized into definite opinion. This is the growing tendency on the part of medical scientific workers, and bacteriologists especially, to patent discoveries of practical therapeutic and public health value. And in approaching this delicate subject I wish to place it on a basis of just appraisal of principles, without creating the false impression that I am assailing any particular group.

Much can be said on both sides of the question, and in discussing it with other bacteriologists and pathologists from time

to time, I can recall opinions of men quite as desirous of maintaining the ethical standards of our profession as I am, who believe that there are circumstances under which the patenting of a laboratory discovery is not only legitimate, but desirable. Indeed, in Germany—a country in which the quality of scientific work accomplished is quite as high as that of any other—such patents have long been in vogue, both for chemical therapeutic agents and biological products. Opinions and precedents, therefore, are not entirely in favor of the view I am about to express.

The general purpose of commercial patent laws is admittedly that of assuring an adequate financial recompense for the enterprise and ingenuity of those who have developed a method or a product. This is a legitimate view in purely commercial undertakings. We have prided ourselves in our profession, however, upon the fact that scientific investigation is not a commercial pursuit, and that discoveries should accrue to the benefit of the community. This attitude cannot be regarded as based on ethical considerations alone, if we remember that most of the institutions in which we work and the endowments which furnish equipment, materials and training have been provided either by private benevolence or by the sense of public obligation acknowledged by universities for the precise purposes of furthering the progress of knowledge. On this basis alone the financial argument, legitimately applicable to purely commercial undertakings, breaks down when we attempt to apply it to scientific discovery. The ethical argument remains a strong one, but it is perhaps unwise to base this discussion entirely upon it, since ethical questions are open to individual differences of interpretation.

Leaving aside the commercial point of view, then, let us examine the practical arguments which may be advanced in favor of patents, and consider to what extent they may be considered valid.

In one instance which I recall, the argument advanced was that, by a reasonable patent, taken in the name of the institution rather than of the individual, the exploitation of a commercially valuable procedure by private firms could be regulated. This

attitude, arising from a sincere motive, would seem to us utterly untenable, since, without a patent, cheapness of production would eventually be an inevitable result of competitive manufacture, and the existence of a patent would merely act as a price-fixing device, unnecessary and artificial, however wisely applied.

Another basis upon which it is said that patents are usually taken is that of controlling early distribution for two main reasons.

One of these concerns the desire to prevent erroneous clinical conclusions which might accrue from the use of preparations of insufficient potency by physicians who are not adequately trained in the use of the particular type of therapy involved. This, it is said, might delay the general acceptance of the new method and its proper application to appropriate cases. There would be some justice in such an argument were the organization of our laboratories and the education of our physicians less sound. It seems self-evident, however, that any discovery of great practical importance, once published with adequate details, would be widely re-investigated in many laboratories by competent men under the guidance of the discoverers, and the possibility of error would be diminished rather than increased.

Closely related to this motive alleged in favor of controlling early production is the just desire to safeguard the quality of the product in such a manner that the public may be protected from the indiscriminate distribution of inactive materials insufficiently controlled in regard to safety.

This responsibility would rest upon the discoverers only if the United States Public Health Service were not admittedly a perfectly effective agency for protecting the public in these respects, an instrument far more effective than anything that can be accomplished by patents.

Against these few doubtful advantages many objections against the practise of patenting scientific discoveries can be advanced.

If the patent concerns a method, it immediately limits further investigation, and we know by experience that modifications and improvements in biological procedures can only be obtained by free experimentation. Such limitation also interferes with the

prompt and free application of the method by the numerous departments of health of the states and cities, which are under public obligation to develop as rapidly and effectively as possible any advances in biological diagnostic or therapeutic methods which promise to benefit the communities they serve.

If the patent concerns a fundamental principle, it is manifestly an absurdity, because no serious worker in a biological subject will neglect the application of a new fundamental principle to a different line of thought because of the fear of being sued for infringement. The influence on the younger workers, moreover, is to encourage the desire to occupy themselves with procedures that offer prospects of practical application, when often their natural inclinations might impel them to devote themselves to fundamental questions.

The atmosphere created by the habit of applying for patents, moreover, is apt to create a spirit of competition and haste where there should be detachment and contemplation. From the point of view of pure justice, moreover, it should never be forgotten that no discovery in biological medicine has ever been made which has not been based upon the cumulative heritage of previous observers. The final step by which these observations are transformed into a practically applicable method, however ingenious and clever, is after all nothing more than the last link in a long chain of progress, every part of which has contributed almost as much as the last one to the final achievement.

All these are practical considerations and seem to us sufficiently important to call for serious discussion by a group of workers whose association is based upon the desire to further the interests of our science.

It should not be necessary, in addition to these arguments, to invoke the ethical considerations which seem to many of us to be incompatible with the patenting of principles or methods involved in the maintenance of individual or public health. It seems to us probable that no code of ethics has ever developed from purely abstract considerations, and that in every case where a strong feeling of propriety of action has been transformed into a tradition of behavior, there has been behind it some tangible

purpose. Standards of behavior which have developed in all things connected with medicine have sprung from a recognition that there is a sharp line of differentiation between those forms of activity which deal with health, and those which are purely commercial. The invention of an improvement in the mechanism of automobiles, or of a shoe-buckle, concerns matters of convenience or luxury, and can be dispensed with easily by those who are forced to do without them. The relief of the sick and the prevention of unnecessary sorrow by the maintenance of individual and public health are matters in a different category. As soon as we are in possession of the knowledge of principles or methods which can contribute to these purposes their free utilization becomes a public necessity; and any procedure which inhibits their most rapid and effective application to the needs of the community would seem to us as unjustified as the cornering of the wheat market or the patenting of the process of making bread. The principle, it seems to us, is the same, though any examples which are derived from recent occurrences in medicine are less extreme. Let us suppose, however, that Jenner had patented vaccination, or that patents had been taken out on the methods of staining the tubercle bacillus,—the very hardships that this might have threatened would have invalidated the patent claims by community disapproval.

If patents have been taken and successfully exploited in any important medical procedures, they have either been so limited in their applications that the situation has been allowed to continue without much comment, or else they have been so modified and adjusted that the community has not been allowed to suffer. This does not alter the principles involved.

Altogether, it seems to us quite apparent that there is no valid argument in favor of the patenting of a useful method of preserving health, private or public, unless we admit that medical discoveries in no ethical sense differ from the purely commercial ones. We ourselves are not willing to admit this, but if there are others who believe that this is a defensible attitude, let us be done with the sophistry of other arguments in favor of the patents and frankly admit that they are taken out on this purely commercial basis.

It must not be forgotten, moreover, that there are rewards in the successful accomplishment of scientific medical discovery quite absent from any commercial undertaking, and far more valuable. When a splendid piece of work such as one that has recently been patented has been accomplished—the result of years of painstaking and accurate observation, and an instrument of incalculable good in terms of comfort, relief of suffering and the saving of life—it seems a pity that considerations which place it in the category of commercial inventions should detract from the fineness of the achievements and from the pride which we would like to take in those who have achieved them.

STUDIES UPON THE RELATIONSHIP BETWEEN SURFACE TENSION AND THE ACTION OF DISINFECTANTS, WITH SPECIAL REFERENCE TO HEXYLRESORCINOL

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I. ADDITION OF SURFACE TENSION DEPRESSANTS TO DISINFECTANT SOLUTIONS

It has been previously demonstrated by Leonard (1924) and his colleagues that hexylresorcinol, when taken by mouth in therapeutic doses, is excreted by the kidneys and appears in the urine in sufficient concentration to kill bacteria in large numbers. A discussion of hexylresorcinol as a urinary antiseptic has been published elsewhere (Leonard, 1925).

It has been shown by Leonard (1923) that: the administration to persons taking hexylresorcinol, of sodium carbonate, robs the urine of its bactericidal power. It was shown that neither the alkalinity of the urine nor the presence of soda had any direct effect on the activity of the hexylresorcinol. It was found, however, that the administration of hexylresorcinol in therapeutic doses resulted in the secretion of urine of very low surface tension, while the administration of soda gave rise to urine of very high surface tension.

It was therefore suggested that surface tension might play some rôle in governing the activity of hexylresorcinol in urine. Four of the chief theoretical considerations involved in this idea may be summarized briefly as follows:

1. Fluids of very low surface tension may, under some circumstances, permeate or penetrate into minute crevices or

interstices more readily than fluids of high surface tension. The laws governing capillary rise and the limitations due to viscosity must, of course, be considered when interpreting this generalization.

2. Osmosis and diffusion are known to be materially accelerated under certain conditions (Billard and Dieulafe, 1904) by lowering the surface tension of the solutions of fluids involved.

3. Disinfectants which lower surface tension are adsorbed on the surfaces of the organisms to be killed and therefore exist in higher concentration at the most effective point. This reaction is common to surface tension reducents in general, germicidal or otherwise, in response to the law of entropy.

4. It is a matter of common experience that heat tends to dissociate adsorption complexes or decrease the extent of adsorption. Conversely, a surface tension reducent will be adsorbed to a greater extent at low temperature than at high. Experimentally, this has been demonstrated by Bayliss (1924). Therefore, of two disinfectants having equal value at a given temperature, of say 40°C., the one which lowers surface tension most powerfully should retain its efficiency to a greater extent when the temperature is lowered to, say 20°C., since adsorption of the stronger surface tension reducent, with resulting concentration about the bacterial cell should occur to an increasing degree as the temperature is lowered.

It would appear therefore that surface tension may play a very important rôle in disinfection by chemical means, as well as in other cellular processes. Experimental evidence in support of the view is to be found in the literature. The work of Berczeller (1924), Hansen (1922), Billard (1906), Liese and Mendel (1923), Frobisher (1926), Leonard and Frobisher (1926), Bechold and Reiner (1922) and others may be cited in this connection.

By comparative tests *in vitro* hexylresorcinol was shown to be practically as powerful a reducent as chemically pure sodium oleate (Leonard and Frobisher, 1926). The combination of very great bactericidal activity and powerful surface tension reducent properties exhibited by this substance naturally sug-

gested its use in further investigation of the significance of surface tension factors in chemical disinfection.

EXPERIMENTAL

From the theoretical considerations and experimental data set forth above, the question arises whether one can increase the bactericidal activity of a disinfectant by lowering the surface tension of its solution through the addition of a non-bactericidal reducent.

In all tests of bactericidal power mentioned in this paper, the method detailed by the United States Hygienic Laboratory (1921) was used as a basis. The only departures from the prescribed technic were in the use of ordinary (not certified) pipettes and in the substitution of Difco pepton for Armour's in preparing the culture medium. Certain experimental modifications of the methods were made, but these are described in connection with the experiment involved.

The measurements of surface tension were made by placing 1 cc. of the described solution in a chemically clean watch glass and allowing this to stand undisturbed for exactly two minutes. At the end of this time the loop of a du Nouy tensiometer (1919) was brought into contact with the fluid, the reading made immediately and then checked twice. In making mixtures for the measurement of surface tension the total volume of fluid in all tubes was equalized with sterile distilled water. All measurements of surface tension were made at temperatures between 19°C. and 21°C.

All solutions of reducent were examined for contamination at the time of each experiment and were found to be sterile in every case.

The organism (*Bacillus typhosus*) used in the experiments to be described has been shown to be affected little, or not at all, by low surface tensions (Frobisher, 1926). The apparent superiority of disinfectant solutions of low surface tension in these experiments can, therefore, hardly be ascribed to a susceptibility of the test organism to this physical factor alone.

The surface tension depressants used in these studies have

previously been shown (Frobisher, 1926) to have little or no effect on *Bacillus typhosus*. It is also obvious in the experimental data presented below, that the effects obtained have not been the result of any toxic action of the depressants themselves.

TABLE 1

Effect of a small quantity of sodium oleate upon the bactericidal power of phenol

PHENOL DILUTIONS AND THEIR SURFACE TENSIONS			TURBIDITY OF SUBCULTURES					
			No sodium oleate			0.1 cc. of 1 per cent sodium oleate		
Dilutions	S.T.* no oleate	S.T.* oleate, 0.1 cc. 1 per cent solution	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
80	66.4	42.2	−	−	−	+	−	−
90	68.0	42.2	−	−	−	+	−	−
100	69.5	40.6	+	−	−	+	−	−
110	70.3	41.4	+	+	−	+	+	−
120	71.1	41.4	+	+	+	+	+	+

* Surface tension, dynes per centimeter.

TABLE 2

Effect of a moderate quantity of sodium oleate upon the bactericidal power of phenol

DILUTIONS OF PHENOL			TURBIDITY OF SUBCULTURES					
			No sodium oleate			0.25 cc. of 1 per cent sodium oleate		
Dilutions	S.T.* no oleate	S.T.* oleate, 0.25 cc. 1 per cent solution	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
90	68.0	39.2	−	−	−	−	−	−
100	69.5	37.5	−	−	−	−	−	−
110	70.3	39.2	+	−	−	−	−	−
120	71.1	39.2	+	+	−	−	−	−
130	71.9	39.2	+	+	+	+	+	−

* Surface tension, dynes per centimeter.

In the first experiment a series of dilutions of phenol was freshly prepared in duplicate and 0.1 cc. of a sterile 1 per cent solution of sodium oleate was added to each of the dilutions of one set. The surface tension of each dilution of both sets was then meas-

ured and the standard technic proceeded with. The results are recorded in table 1.

Because the result of this experiment seemed rather inconclusive, the test was repeated using 0.25 cc. of oleate solution in place of 0.1 cc. as before. The result is shown in table 2. This experiment seemed to show a definite superiority in bactericidal power of the phenol mixtures having the lower surface tension. The results of a similar experiment using 0.5 cc. of the oleate solution are shown in table 3.

The seemingly contradictory result of the third experiment appears readily explicable when we consider the nature of the

TABLE 3

Effect of an excess of sodium oleate upon the bactericidal power of phenol

DILUTIONS OF PHENOL			TURBIDITY OF SUBCULTURES					
			No sodium oleate			0 5 cc of 1 per cent sodium oleate		
Dilutions	S T.* no oleate	S T.* oleate, 0 5 cc. 1 per cent solution	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
90	68 0	37 5	–	–	–	+	+	+
100	69.5	39 5	–	–	–	+	+	+
110	70.3	39 2	+	–	–	+	+	+
120	71.1	39 2	+	+	+	+	+	+
130	71.9	39 2	+	+	+	+	+	+

* Surface tension, dynes per centimeter.

sodium oleate. This soap is not only a much more powerful reducent than phenol, but it is comparatively inert against the typhoid bacillus. Furthermore, soap, even when containing appreciable quantities of water, is a solid or semi-solid at ordinary temperatures. It is known that when two surface tension reducents are present in a solution, the more powerful will displace the weaker at the adsorption surfaces. It is believed, therefore, that in the last experiment described above the surface of the bacteria, when introduced into the soap-phenol solution, became coated with a film of sodium oleate concentrated to the extent of being more or less solid (Ramsden, 1904)

under the influence of adsorption and that this film acted as a protective covering, preventing contact of the phenol with the cell. The results of Hansen (1922) who reported an inhibitory effect by saponin and soap when added to disinfectant solutions seem to be in agreement with this explanation.

A fluid surface tension reducent was therefore sought which, when adsorbed, might still remain miscible with the phenol and oppose no solid barrier to its diffusion into the cell. Ethyl acetate was selected as meeting these requirements. Ethyl acetate has previously been shown (Frobisher, 1926) to be non-toxic for *Bacillus typhosus* in low concentrations. Two sets of the usual dilutions of phenol were made as before, one containing no ethyl acetate and each tube of the other set receiving 0.1 cc. of this substance. As controls, two tubes containing 5 cc. of distilled water were prepared and to them were added 0.1 cc. and 0.25 cc. of ethyl acetate, respectively. After measuring the surface tension of all mixtures, the standard method was proceeded with as usual. Subcultures from the control tubes

TABLE 4

Effect of ethyl acetate upon the germicidal power of phenol

DILUTIONS OF PHENOL			TURBIDITY OF SUBCULTURES												
			No reducent			0 1 cc of E. A.†			Controls						
Dilutions	S.T.* no E. A.†	S.T.* E. A †	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes	20 minutes	25 minutes	30 minutes	35 minutes
80	66.4	56.3	–	–	–	–	–	–							
90	68.0	56 3	+	–	–	–	–	–							
100	69 5	56.3	+	+	–	–	–	–							
110	70 3	56.3	+	+	–	–	–	–							
120	71 9	58.6	+	+	+	–	–	–							
Control 0.1 cc. E. A.†									+	+	+	+	+	+	+
Control 0 25 cc. E. A.†									+	+	+	–	–	–	–

* Surface tension, dynes per centimeter.

† Ethyl acetate.

were continued for twenty minutes beyond the usual period. The experiment was repeated with practically identical results. Both are summarized in table 4.

Control number two, in this table indicates that ethyl acetate is not entirely bland. But this control contained 2.5 times as much acetate as any of the other tubes in the experiment and, furthermore, showed no bactericidal action whatever during the time of the test. Control number one indicates that 0.1 cc. of ethyl acetate is not sufficiently toxic to account for the increased bactericidal power of the phenol solutions containing it without taking physical factors into consideration. It is evident that the bactericidal power of solutions of phenol may be greatly increased by reducing their surface tensions from about 70 dynes to about 57 dynes with ethyl acetate. Other experiments, on the contrary, indicate that the bactericidal power of such phenol solutions is not greatly altered even though the surface tensions be lowered from about 70 dynes to about 42 dynes with sodium oleate (table 1). It seems probable that even if, in the experiment represented by table 1, a quantity of sodium oleate had been used sufficient to give surface tensions of about 57 dynes (similar to those recorded in table 4), there would have been no increase in bactericidal power. There is obviously some difference between the action of ethyl acetate and sodium oleate as surface tension reducents.

The complete inhibition of disinfectant action observed in the experiment involving an excess of soap (table 3) is in striking contrast to the marked increase in bactericidal action seen in the experiment involving a small quantity of soap (table 2). Yet the only difference in these two experiments is in the quantity of sodium oleate used. This would seem to lend additional support to the suggestion already made, that where an excess of solid surface tension reducent is added to disinfectant solutions, enough reducent is adsorbed on the surface of the cells to prevent contact of the disinfectant and the cell.

A series of experiments was now undertaken which were, in general, very much like those just described except that hexylresorcinol was substituted for phenol. A preliminary test of

this sample of hexylresorcinol, which happened to be one of exceptional purity, showed it to be active against the Hopkins strain of *Bacillus typhosus* in a dilution of 1:15,000 in five minutes, at 20°C.

In the first experiment two series of dilutions were prepared. To the first series, no reducent was added. To each tube of the second series was added 0.25 cc. of sterile 1 per cent sodium oleate solution.

TABLE 5

Effect of ethyl acetate and of an excess of sodium oleate upon the bactericidal power of hexyl-resorcinol

DILUTIONS OF HEXYL-RESORCINOL				TURBIDITY OF SUBCULTURES								
				No reducent			0.5 cc. of 1 per cent S.O.‡			0.1 cc. E.A.		
Dilutions	S.T.,* E. A.†	S.T.,* oleate	No reducent	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
10,000	53.1	35.9	58.6	−	−	−	+	+	+	−	−	−
11,500	54.2	36.7	59.0	−	+	−	+	+	+	−	−	−
13,000	56.3	36.7	61.7	+	+	−	+	+	+	−	−	−
14,500	56.3	36.7	62.5	+	+	+	+	+	+	−	−	−
16,000	59.4	36.7	64.1	+	+	+	+	+	+	−	−	−

* Surface tension, dynes per centimeter.
† Ethyl acetate.
‡ Sodium oleate.

The usual technic was then employed in comparing the bactericidal strengths of these two series of dilutions. As in the experiments with phenol, it was found that this quantity of sodium oleate definitely increased the efficiency of the hexylresorcinol solutions containing it. A second experiment was then made involving three series of suitable dilutions of hexylresorcinol. The first series contained no reducent. To each tube of the second series was added 0.5 cc. of 1 per cent sodium oleate solution while each tube of the third series received 0.1 cc. of ethyl acetate. The results obtained by proceeding with the standard method in each case are shown in table 5.

It is seen that the bactericidal power of hexylresorcinol is affected by surface tension reducents in a manner similar to that in which phenol is affected.

A practical application of these results might well be made in the use of disinfectants in association with soap. Many mixtures of ordinary soap are made with various germicidal substances in which the optimum quantitative relationship between soap and germicide is apparently given little or no consideration. The efficiency of these mixtures might in many cases be greatly increased by a more careful adjustment of this relationship.

The futility of mixing small quantities of disinfectant with soap is indicated by some experiments recently performed by Hampil in the Department of Bacteriology of the Johns Hopkins School of Hygiene and Public Health. This worker made determinations of the germicidal power of hand soaps especially prepared for this work and containing 1 per cent, 2 per cent, and 3 per cent pure hexylresorcinol, respectively. In the first series of tests, dilutions ranging from 1:50 to 1:100 were tried at 20°C. No germicidal power was noted with any of the soaps. At the suggestion of the author, greater dilutions were used and the temperatures raised from 20° to 37°C. It was hoped that dilution and higher temperature would combine to lessen the degree of adsorption and solidification of the soap on the bacterial surfaces so that the hexylresorcinol would be enabled to act more freely. In the dilutions used hexylresorcinol, at 37°C. would ordinarily be highly fatal for the test organism used, (*Bacillus typhosus*). It was found that no germicidal action whatever occurred under either of the conditions outlined above.

It might be concluded that the addition of ethyl acetate or suitable amounts of sodium oleate and possibly of other surface tension reducents to disinfectant solutions would not only enhance the activity of solutions already effective, but would also tend to render active, dilutions which would otherwise be devoid of bactericidal properties.

The latter suggestion brought up the question as to how far one might dilute hexylresorcinol and still have a solution, ineffective by itself, but effective within fifteen minutes at 20°C., provided the surface tension were lowered.

TABLE 6

Effect of ethyl acetate upon the bactericidal power of higher dilutions of hexylresorcinol

Readings based upon turbidity of subcultures after fifteen hours incubation

DILUTIONS OF HEXYLRESORCINOL			TURBIDITY OF SUBCULTURES					
			No reducent			0.1 cc. of 1 per cent ethyl acetate		
Dilutions	S.T.,* E.A.†	S.T.,* no E.A.†	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
15,000	57.8	62.5	−	−	−	−	−	−
18,000	58 6	65 6	+	−	−	−	−	−
21,000	61.7	67.2	+	+	±	±	−	−
24,000	62.5	68.2	+	−	+	−	−	−
27,000	62.5	68.8	+	+	+	−	−	−
30,000	64.1	70.3	+	+	+	±	−	−
33,000	63.3	71.1	+	+	+	+	−	−
36,000	64.1	72.7	+	±	+	+	+	±
39,000	64.1	74 2	+	+	+	+	+	+
42,000	64.1	75.5	+	±	±	+	+	+

* Surface tension, dynes per centimeter.

† Ethyl acetate.

TABLE 6A

Effect of ethyl acetate upon the bactericidal power of higher dilutions of hexylresorcinol

Readings based upon turbidity of subcultures after forty-eight hours incubation

DILUTIONS OF HEXYL-RESORCINOL	TURBIDITY OF SUBCULTURES					
	No reducent			0.1 cc. of 1 per cent ethyl acetate		
	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
15,000	+	+	+	−	+	−
18,000	+	+	+	+	−	−
21,000	+	+	+	+	+	+
24,000	+	+	+	+	±	+
27,000	+	+	+	+	+	+
30,000	+	+	+	+	+	+
33,000	+	+	+	+	+	+
36,000	+	+	+	+	+	+
39,000	+	+	+	+	+	+
42,000	+	+	+	+	+	+

To answer this an extreme range of dilutions was made and divided into two series as before, one set of dilutions receiving 0.1 cc. of distilled water and the other series 0.1 cc. of ethyl acetate. The usual technic was then applied. The results are shown in tables 6 and 6a.

The results given in table 6 are based upon readings made after fifteen hours incubation of the subcultures. The + signs indicate a definite turbidity due to good growth. The ± signs indicate a possible turbidity, so slight as to render a definite reading impossible.

It is interesting to compare the results of the same experiment read after forty-eight hours incubation of the subcultures. As shown in table 6a, ethyl acetate definitely increases the effectiveness of the higher dilutions of hexylresorcinol but not to the great extent indicated by the results recorded in table 6. The organisms subjected to this treatment seem to have been so altered or injured that they have a tremendously increased lag period or initial growth phase (Buchanan, 1918) or both. The nature of the injury producing this lag is obscure. There is reason to believe that the surface tension reducent induces changes in the permeability of the membrane which facilitate the entrance of the hexylresorcinol *into* this membrane. It may be that the injured membrane remains impregnated with a certain sublethal amount of hexylresorcinol. This, transmitted to the daughter cells as they form, would continue to injure these cells, possibly impeding growth long after the parent cells had been removed from the seeding tubes, thus producing an extended lag period. It does not seem likely, in the experiment under discussion, that the delay in appearance of turbidity was due to the fact that only small numbers of bacteria were transferred to the subculture since readily visible turbidity will appear in fifteen hours when only one or two normal living cells are transferred to such medium.

There is always the possibility that a compound may be formed by a reaction between the surface tension reducent and the germicide under investigation, which is a stronger germicide than either of these substances alone. The experiments recorded, in-

dicate on the contrary a very close relationship between germicidal power and low surface tension. The possibility of the formation of a new and more germicidal compound seems to be lessened by the fact that identical results may be obtained with substances that are quite dissimilar chemically.

The experiments described above appear to justify the conclusion that it is possible to increase or decrease the effectiveness of phenol and of hexylresorcinol by appropriate changes of the surface tension of their solutions with *suitable* substances.

II. RÔLE OF SURFACE TENSION WHEN THE SUBSTANCE INVESTIGATED IS AT ONCE DISINFECTANT AND SURFACE TENSION REDUCENT

A second type of experiment was undertaken in the hope of shedding some light on the rôle of surface tension in disinfection when the surface tension reducent is the disinfectant itself instead of a more or less inert secondary substance. The question to be answered might be put as follows: Are substances which reduce surface tension more powerful disinfectants than those which do not? The behavior of certain substances seems to provide a negative answer. For example, sodium oleate is a powerful reducent but is only slightly or not at all germicidal (against *Bacillus typhosus*) while a solution of $HgCl_2$ which has a high surface tension, is a powerful germicide. This might indicate that low surface tension can play little or no rôle in disinfection. It is true that $HgCl_2$ and many other similar inorganic disinfectants do not produce any considerable change in the surface tensions, which is measurable at the air-water interface. It has been shown by Hansen that surface tension may nevertheless play a very distinct rôle in the action of even this type of disinfectant, and that the efficiency of such disinfectants can be increased by adding surface tension reducents to their solutions. As regards the lack of germicidal value of soap[1] it

[1] Sodium oleate in its action against *Bacillus typhosus* is referred to here. The work of Walker (1924 and 1925) has shown that many soaps may be germicidal and that organisms differ in their resistance to the action of soaps. It is suggested that the more germicidal soaps in Walker's series may be the more powerful surface tension reducents. No data are given on this point.

is to be pointed out that ability to lower surface tension should not be regarded as the only factor in *determining* whether or not a substance is germicidal. It seems obvious that substances like soap,[1] sugar or pepton, showing no chemical germicidal action can not be made germicidal merely by reduction of the surface tension of their solutions. Given a substance, however, which exhibits even a slight tendency to kill bacteria by reacting with them chemically, it is believed, in light of the data given above and found in the literature, that by altering the surface tension of solutions of such a substance it may be made more or less efficient according to whether the surface tension of the solutions is lowered or raised. The rôle of the surface tension reducent in such a case appears to be to facilitate contact between the substance and the cell or cell contents.

Further remarks, with a suggested explanation as to why one good disinfectant fails to lower surface tension while another seems largely dependent on its reducent properties, are given under the heading, "Discussion."

Confining our attention for the present to the rôle of surface tension in the activity of the phenolic type of disinfectant, it should be found, theoretically, that those which lower surface tension most powerfully are the better disinfectants. The facts, as applied to chemically unrelated phenol derivatives, may not follow the theory strictly. But if we compare the members of an homologous series of compounds, it should be found that increased ability to lower surface tension is coincident with increased bactericidal efficiency.

The availability of resorcinol together with an unusually complete homologous series of both its acyl- and alkyl-derivatives, all in pure crystalline form, offered a good opportunity to make this comparison.

The phenol coefficient of each of these substances was determined by the standard method and checked several times. The surface tension of a suitable dilution of each was measured and the results of both series of determinations have been tabulated in parallel columns (tables 7 and 8).

It is interesting to note that in both series strict parallelism

TABLE 7

Relation between the bactericidal power of the alkyl resorcinols, their ability to reduce surface tension and the weight of the side chain

COMPOUND	SUM OF THE ATOMIC WEIGHT OF THE ATOMS IN THE ALKYL CHAIN		PHENOL COEFFICIENT		SURFACE TENSION 1:10,000 DILUTION (DYNES PER CENTIMETER)	
	Normal compounds	Iso compounds	Normal compounds	Iso compounds	Normal compounds	Iso compounds
Resorcinol.....	—		0.3		76	
n-propyl.. ..	42		5		73	
n-butyl......	57		22		66	
iso-butyl....		57		15		65
n-amyl......	71		33		60	
iso-amyl.....		71		24		62
n-hexyl......	85		50+		54	
iso-hexyl....		85		27		56
n-heptyl.....	99		30		43	
n-octyl......	113		0		27(?)*	
n-dodecyl....	169		0		49(?)*	

* Very turbid.

TABLE 8

Relation between bactericidal power of the acyl-resorcinols, their ability to reduce surface tension and the weight of the side chain

COMPOUND	SUM OF ATOMIC WEIGHTS OF ATOMS IN SIDE CHAINS		PHENOL COEFFICIENT		SURFACE TENSION 1:6500 DILUTION (DYNES PER CENTIMETER)	
	Normal compounds	Iso compounds	Normal compounds	Iso compounds	Normal compounds	Iso compounds
Resorcinol.....	—		0.3		71	
n-propylyl ..	57		1±		68	
n-butylyl....	71		16		59	
iso-butylyl..		71		3		67
n-amylyl....	85		33		57	
iso-amylyl...		85		1±		77
n-hexylyl....	99		65+		53	
iso-hexylyl..		99		†		47
n-heptylyl...	113		*		41	
n-octylyl....	127		*		47‡	
n-dodecylyl .	183		*		49‡	

* Not soluble at 20°C.
† Not determinable; insoluble.
‡ Very turbid.

between bactericidal power and ability to lower surface tension obtains up to a certain point. With an increase in the number of carbon atoms in the alkali chain beyond six or seven, the bactericidal power falls off very markedly although ability to lower surface tension is not diminished. It is interesting that the isomeric compounds form a sort of separate series in which the parallelism between bactericidal power and ability to lower surface tension is maintained but without relation to the normal compounds.

SUMMARY, DISCUSSION AND CONCLUSIONS

In the foregoing pages, data have been presented in support of the view that low surface tension plays an important rôle in disinfection. Two types of experiment have been performed. In one series the surface tension of the disinfectant solutions was lowered by the addition of a germicidally inert secondary substance, and in another the disinfectant itself lowered the surface tension. In the former type it was found that if care be taken to use secondary substances of the proper sort and in suitable quantities the germicidal efficiency of both phenol and hexylresorcinol against *Bacillus typhosus* at 20°C. may be increased by lowering surface tension.

The secondary substances used were sodium oleate and ethyl acetate. It is to be noted that neither of these substances partakes to any great extent of the nature of an electrolyte. The addition of electrolytes to disinfectant solutions is known, in many cases, to alter their efficiency without producing any marked change in the measurable surface tension of the solutions (Paul and Krönig, 1896 and Bial, 1902). This is attributed by some workers (Bial, 1902 and Norton and Hsu, 1916) to the effects of various ions upon each other. Penetration of ions through semipermeable membranes can be facilitated in some cases by introducing certain other ions into the solutions involved (Philips, 1913). The results are thought to be due to neutralization of electrical charges on the ions which permeate.

The alteration in the efficiency of phenol solutions brought about by the addition of salt may not be due to ionic effects,

however, but to changes in the solubility of the phenol. It has been shown by Paul and Krönig (1896), and Scheurlens (1895) that, by adding inorganic salts to phenol solutions, the efficiency of the solution is greatly increased. It is suggested that as the salt goes into solution, the phenol tends to go out, so that, as far as the bacteria are concerned, the phenol is much more concentrated. Such a process has been described by McClendon (1911) as due to attraction pressure of the solutes.

The question arose, during the course of the work described in this paper, as to why, if surface tension plays so important a rôle in disinfection, $HgCl_2$ is a powerful germicide, but a poor reducent, while sodium oleate, which is a powerful reducent, is a relatively poor germicide against *Bacillus typhosus*. In comparing disinfection by non-reducents such as $HgCl_2$ or HCl with that by good reducents such as phenol, it may be pointed out that, in the case of the heavy metal salts and acids, the action of ions has long been regarded as important. Paul and Krönig (1896), Bial (1902), Winslow and Lochridge (1906) and others have shown that the disinfecting power of certain acids is approximately proportional to the concentration of hydrogen ions. This may not always be true since acetic acid, which is not very extensively ionized as compared with HCl, is, nevertheless, a better disinfectant at the same hydrogen ion concentration (Winslow and Lochridge, 1906). It is significant that acetic acid has a more profound action on the surface tension than has HCl. There is evidence (Paul and Krönig, 1896; Bial, 1902, Norton and Hsu, 1916) that the anion also plays an important rôle in acid disinfection. In the case of formic acid (Norton and Hsu, 1916) the anion is believed to act by influencing the cation in some way. Disinfection by substances such as $HgCl_2$ is said to be in proportion to the concentration of the metallic ion and not of the salt (Dreser, 1893). This may not be true in all cases since $Hg(NO_3)_2$ is a much less effective germicide (Paul and Krönig, 1896) than $HgCl_2$ although the former is much more highly ionized.

In disinfection by substances of the classes represented by $HgCl_2$ and HCl the ions are regarded as the active agents and

efficiency has been regarded as more or less dependent on the concentration of various ions and their effects on each other. How is the efficiency of phenolic compounds to be associated with ionic phenomena when such compounds ionize very weakly as compared with the acids and salts above mentioned? It would appear that the activity of hexylresorcinol and its derivatives must depend on some other property.

In the case of all disinfectants, contact of the active agent with the cell is obviously a necessary preliminary to disinfection, but the forces which operate to bring about this contact may differ quantitatively, qualitatively or in both respects, depending on the type of disinfectant. For example, bacteria and colloidal particles carry electrical charges. They wander in the electric field to one pole or the other. The surfaces of such particles are, therefore, the seat of free electrical energy. They are also the seat of free mechanical energy arising from the surface tension of the fluid in which they are suspended. If oppositely charged particles or ions can, by moving to such surfaces establish an equilibrium by reducing the amount of free *electrical* energy, they will do so in obedience to the second law of thermodynamics. If these particles or ions also have the power of reducing the amount of free *mechanical* surface energy by virtue of an ability to lower surface tension, then they would also come under the influence of mechanical as well as of electrical adsorption forces. A disinfectant which does not ionize extensively is not influenced to a large extent by electrical forces and is to a much greater extent dependent upon mechanical adsorption, and therefore, upon its own power of reducing surface tension. For example, acetic acid would be absorbed electrically to a considerable extent by virtue of its dissociation, but, because of its power to lower surface tension, would also be mechanically adsorbed. HCl is more dependent upon electrical adsorption and less upon mechanical adsorption. Being highly ionized it is a good disinfectant notwithstanding its inability to lower surface tension. Phenol and its derivatives on the other hand, not being as highly ionized depend largely upon their ability to lower surface tension in order to be adsorbed.

In connection with the type of experiment described in the first part of this paper, it may be of interest to refer again to the work of Hansen (1922). This author studied the effect of numerous surface tension reducents upon the bactericidal power of a variety of disinfectants. He obtained his best results with fluid reducents and obtained no increase and sometimes a decrease in bactericidal power when soap and saponin were used as reducents. He might have secured different results had he used quantities of these reducents more nearly approaching the optimum. His results in general support the views outlined in this paper. It is also of interest to note the observation by Paul and Krönig (1896) that the effectiveness of $HgCl_2$ or of $AgNo_3$ is increased by the addition of ethyl alcohol to their solutions.

Billard (1904) has also presented data in support of these views. He has demonstrated that solutions of curare may be made more toxic by lowering their surface tension to an optimum point with a variety of reducents. This worker also found that, in the case of soap as a reducent, by exceeding a certain optimum the action of the curare was interfered with. More recently this worker has shown (1926) that the toxicity of urine for *Daphnia pulex* varies inversely as the surface tension. It is possible, however, that this represents simply an inability on the part of *Daphnia pulex* to resist low surface tension and does not necessarily represent a true toxicity of the urine *per se*.

In connection with the experiments outlined in the second part of this paper, the results of Berczeller's studies (1914) are of interest. This worker investigated a number of rather short series of compounds with very consistent results with which the ideas expressed in this paper agree perfectly.

The data presented in this paper seem to warrant the following conclusions:

1. The bactericidal powers of phenol and of hexylresorcinol, against *Bacillus typhosus* may be enhanced by reducing the surface tension of their solutions by the addition of suitable quantities of sodium oleate or ethyl acetate.

2. An excess of sodium oleate, when added to phenol or hexyl-

resorcinol, inhibits the bactericidal action of the latter substances. This is believed to be the result of the adsorption of the soap on the surfaces of the cells, with the formation of a protective film or coating.

3. The addition of small amounts (up to 3 per cent) of hexylresorcinol to at least one type of commercial hand soap is shown to lend the soap no additional power against *Bacillus typhosus* at 20°C. or 37°C.

4. A direct relation exists between bactericidal power and ability to lower surface tension in two series of homologous resorcinol derivatives, including compounds ranging from resorcinol to dodecyl- and dodecylyl-resorcinol and including both normal and isomeric compounds and numbering 19 substances in all.

It is suggested as a possibility, based upon the data to be found in the literature upon the subject, and upon the data here set forth, that conclusions 1, 2 and 3 above, may apply to the relationship between soaps or surface tension reducents and germicides generally.

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A MODIFICATION OF THE KLIGLER LEAD ACETATE MEDIUM

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The development of a single medium which will afford a maximum amount of information in a minimum time has been the aim of bacteriologists for many years. This has been especially desirable for the differentiation of the colon-typhoid-dysentery group. The need for such a medium and the limited success of the proposed procedures is evidenced by the numerous and varied plating methods described for the isolation of typhoid and dysentery bacilli.

In 1911 Russell (1911) devised a differential medium for use in test tubes. This medium was composed of nutrient agar to which he added 1 per cent lactose, 0.1 per cent glucose and a sufficient amount of 5 per cent aqueous solution of litmus to give the medium a distinct purple violet color. The object of the Russell medium was to obtain a rapid differentiation between the lactose fermenting and non lactose fermenting gram negative bacilli. The introduction of this medium marked a distinct step in the advancement of our knowledge of the gram negative aerogenic bacilli. Today in most of the laboratories of this country it has been adapted, either in its original or modified form, as one of the standard laboratory culture mediums.

In 1916 Kligler (1916) described a medium which gave even more information than did that of Russell. The essential differences between the two were that Kligler omitted the glucose, added 0.05 per cent lead acetate and substituted Andrade's indicator for the litmus in the Russell medium. At the conclusion of this article and in a subsequent one Kligler (1918) stated that

this medium might be successfully combined with the Russell double sugar tube. By the use of the Kligler medium, one is able not only to differentiate between the members of the colon and paratyphoid groups but also to differentiate, with a fair degree of accuracy, between paratyphoid A and B and between typhoid and dysentery strains. Soon after the appearance of Kligler's first article, Jordan and Victorson (1917) published the results of their work on the differential value of a lead acetate medium.

Kligler recommends the use either of meat infusion agar or beef extract agar as a basis for this medium, although he states that sharper reactions are, as a rule, obtained with the meat infusion agar. The agar is adjusted to pH 7.4 or made neutral to Andrade's indicator, 1 per cent by volume of this indicator is added, and the medium is tubed in 5 cc. quantities and sterilized. A lactose-glucose solution containing 20 per cent lactose and 2 per cent glucose is sterilized separately and 0.25 cc. added in a sterile manner to each tube. Both the lactose and the lead acetate solutions may be added before slanting and as soon as the agar is cooled to about 60°C. If this is not done the lead flocculates the pepton.

This medium has been used fairly extensively and has proven in many ways to be valuable for differential purposes. It has not, however, received as widespread use for teaching purposes and in public health laboratories as its usefulness warrants; and in all probability this is due to the time consumed in its preparation and the difficulty in obtaining a clear medium. The investigations herein reported were made in an attempt to overcome these disadvantages.

Experiments were made with regard to (1) various indicators; (2) the optimum temperature for mixing the ingredients of the medium and (3) the best method for its sterilization.

INDICATORS

The question of a more suitable indicator has been taken up by Wight (1925) who reported the use of multiple indicators in the preparation of his "Rainbow Medium." We tried this

modification and found it to be only slightly more valuable in our hands than the original Kligler medium with the Andrade indicator.

We then tested the following indicators singly and in various combinations: Andrade's, phenol red, brom thymol blue and thymol blue. The following formula was used throughout as a basis for the medium.

Bacto-beef extract	5 grams
Pepton (P.D.)	10 grams
Sodium chloride (B. & A.)	5 grams
Agar shreds	15 grams
Tap water	1000 cc.

The agar was first thoroughly washed in running water and was then heated in the required amount of water, until it was dissolved, after which the other ingredients were added. The reaction was adjusted to pH 7.4 and the medium was boiled for from five to eight minutes. At the end of the heating, the reaction was readjusted to pH 7.4 and the sediment allowed to settle to the bottom of the container. The clear supernatant agar was then decanted into another container and was accurately divided into quantities of 100 cc. and sterilized by heating at 10 pounds pressure for twenty minutes.

To each of five flasks containing 100 cc. of the above sterile medium were added 1 gm. of lactose, 0.1 gram of glucose, and 0.05 gram of lead acetate ($Pb(C_2H_3O_2)_2$ Baker's analyzed), the medium having been cooled previously to 50°C. Indicators were then added in the following proportions:

		cc.
Flask 1.	Andrade's indicator	1
Flask 2.	0.02 per cent aqueous solution phenol red	5
Flask 3.	0.04 per cent aqueous solution thymol blue	5
Flask 4.	0.02 per cent aqueous solution phenol red	5
	0.02 per cent aqueous solution brom thymol blue	5
Flask 5.	0 02 per cent aqueous solution phenol red	5
	0.02 per cent aqueous solution brom thymol blue	5
	Andrade's indicator	1

Batches of the above samples were tubed and sterilized at 3 pounds pressure for fifteen minutes, slanted and cooled. This

amount of heat proved sufficient for complete sterilization. Tubes representing the five flasks of medium were then inoculated with different strains of *B. typhosus; B. dysenteriae* Shiga; *B. dysenteriae* Hiss-Russell; *B. dysenteriae* Flexner; *B. paratyphosus* A; *B. paratyphosus* B; *B. aertrycke; B. enteritidis* (Gaertner); *B. typhi-murium; B. morgani; B. proteus; B. fecalis-alkaligenes; B. coli-communis; B. Friedlander; B. acidi-lactici* and *B. lactis-aerogenes*. These tubes were incubated at 37.5°C. and readings were made at the end of twenty-four hours and again at the end of forty-eight hours.

Phenol red used alone proved to be the most satisfactory indicator for the change in pH of the medium and interfered least in the reading of the lead sulphide reaction. The development of acidity produced a canary yellow color, alkalinity a pink, and neutrality no change from the original color of the medium. Organisms producing hydrogen sulphide showed browning of the surface which usually extended along the line of the stab. With this indicator increased acidity caused the yellow color to become lighter in intensity and the browning was more easily distinguished; whereas with the Andrade indicator increased acidity produced a more intense red and obscured somewhat the lead sulphide reaction. This improvement was strikingly demonstrated with cultures of *B. typhosus* which gave a red or acid butt and an unchanged slant with Andrade indicator and a yellow or acid butt and a pink or alkaline slant with phenol red.

Kligler in referring to the work of Sacquépèe and Chevrel (1905) and of Burnet and Weissenbach (1915) on the use of lead acetate for differentiation said, "Neither of these authors say anything about the nature of the lead acetate reaction. At first thought one would expect it to be a reaction between the H_2S liberated and the lead. If that were so, sugar exerting a sparing effect on the utilization of pepton, should inhibit or completely interfere with the reaction. This was found not to be the case. The experiments indicated that the reaction involved is probably a reduction of the lead acetate to lead oxide (PbO_2) which is brown." However he did not state the nature of the experiments by which he reached this conclusion. It was our belief that the reaction was

due to the formation of lead sulphide. To determine this point, strips of white filter paper saturated with lead acetate solution were suspended inside the upper end of the tubes inoculated with *B. typhosus* and the other strains of organisms mentioned above. In every case cultures which showed browning of the lead acetate medium also showed browning of the lead acetate paper, whereas cultures causing no change in the medium produced no change in the paper. The positive papers, when tested chemically, gave positive reactions for lead sulphide and negative reactions for lead oxide (Prescott and Johnson, 1918). We, therefore, believe the browning in both instances to be due to the same process, i.e., the production of hydrogen sulphide by the growing organisms and its interaction with the lead acetate to form lead sulphide.

PREPARATION AND STERILIZATION

The other two points of interest in the problem, namely, the optimum temperature for mixing the ingredients of the medium and the most satisfactory method for sterilizing it can be considered in the same general discussion. It is well known that the indicators and the carbohydrates can be added to a medium at any temperature, without producing unfavorable results. Kligler found that his best results were obtained by adding separately the carbohydrates and lead acetate solution to agar which had been tubed, sterilized and cooled to about 60°C. It is obvious that if it were possible to mix all of the ingredients of the medium in a flask, then tube and sterilize it, one would have a valuable differential medium which could be prepared as easily and simply as extract agar. With this object in view, experiments were carried out as follows:

In each of three flasks were placed 500 cc. of the melted nutrient agar containing 5 grams lactose, 0.5 gram glucose and 25 cc. of 0.02 per cent solution phenol red, prepared according to the formula given above. To each of these flasks 0.25 gram of lead acetate, either crystalline or in solution, was added at the following temperatures and the corresponding changes noted.

Flask No. 1.	Temperature 100°C.	Distinct flocculation.
Flask No. 2.	Temperature 75°C.	Slight flocculation.
Flask No. 3.	Temperature 50°C.	No flocculation.

The medium in each of these flasks was then tubed through sterile funnels into sterile tubes in 5 cc. amounts and sterilized in the following manner. One-third of the tubes from each of the flasks was heated in the autoclave at 5 pounds pressure for twenty minutes; one-third at 10 pounds pressure for fifteen minutes and the remaining third at 15 pounds pressure for fifteen minutes. All tubes were slanted with butts sufficiently deep for making good stab inoculations. It was noted that sterilization was complete in each instance and had not increased the turbidity of the medium.

The various sets of tubes were then inoculated with each of the 16 strains of organisms used in our other experiments, incubated for twenty-four hours, and the reactions in all tests could be read with a fair degree of accuracy. However, the most satisfactory reactions were obtained in those tubes to which the lead acetate had been added at 50°C., irrespective of the temperature used for sterilization.

SUMMARY

The essential points noted by us in the foregoing experiments are:

1. Phenol red gives more satisfactory results as an indicator than Andrade's indicator which is used in the Kligler medium.
2. It is not necessary to add the sterile lead acetate to melted sterile agar tubes, but all of the ingredients of the medium can be mixed in bulk at a temperature of 50°C. or lower thus preventing flocculation. The lead acetate can be added either in crystals or in solution. The medium can then be tubed and sterilized by heating in the autoclave at 5 pounds pressure for twenty minutes.
3. No damage occurs and no flocculation takes place, even though sterilization be accomplished by heating at 15 pounds pressure for fifteen minutes.
4. The browning of the lead acetate medium is due to the for-

mation of lead sulphide and in the absence of this medium, the production of H_2S by organisms may be determined by suspending in the neck of the culture tubes strips of white filter paper saturated with lead acetate solution.

5. The preparation of the medium thus simplified makes it thoroughly practical for use in teaching laboratories and in public health diagnostic laboratories.

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STUDIES ON RESPIRATORY DISEASES

XXIX. THE INFLUENCE OF ANTI-SERUM AND OF ANIMAL PASSAGE UPON THE VIRULENCE AND ELECTROPHORESIS OF PNEUMOCOCCI[1]

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I. INTRODUCTORY

In earlier publications of this series, Falk, Gussin and Jacobson (1925, Falk, Jacobson and Gussin (1925) and Falk and Jacobson (1925, 1926, 1926a) discussed at length the parallel relations between the virulence and the electrophoretic potentials of pneumococci. It was found that the potentials are different for the several types of pneumococci and that the sequence of decreasing potentials (types III, I, II, IV) is identical with the sequence of decreasing virulence for white mice. It was found further that variant strains (Blake) of type I pneumococci which differ in their virulence for mice also differ, in a parallel manner, in their electrophoretic potentials. These differences were demonstrated upon single cell subcultures as well as upon the original strains.

Falk and Jacobson also found for pneumococci, as had already been found for other bacteria, that the electrophoretic potential difference (P.D.) is a function of the pH of the menstruum and that the relative agglutinability—specific (serum) and non-specific (acid-alkali)—is an inverse function of the P.D. The correlation between P.D. and inagglutinability was further evidenced by the inversion of the relative suspension stability of

[1] This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

the *A*. *B*. *C*. strains, tested with agglutinating serum, after they had been washed sufficiently to invert the relative magnitudes of the potentials.

Many investigators have emphasized in recent years the significance of "smooth" and "rough" variations in bacteria. It has generally been found that strains which give "smooth" colonies on suitable media are (relatively) highly virulent and that strains which give "rough" colonies are slightly or not at all virulent when tested in appropriate animals. Under specified conditions, "smooth" strains can be converted to the "rough" varieties (*vide* Reimann, 1925; Amoss, 1925) and "rough" to the "smooth" (Jordan, 1926).

The normal and variant strains of pneumococci upon which we have already reported were all strains which gave "smooth" colonies on pepton, serum and blood agar media. Even the strain *C*, which is ordinarily without virulence for mice, gives "smooth" colonies. If a mouse be killed with a very large dose of these organisms, only virulent organisms of the smooth A variety are recovered.

The experiments reported in this paper were undertaken to determine whether "rough" colony varieties of these pneumococci could be produced, and whether changes in virulence would be associated with alterations in P.D. We also undertook a series of experiments to determine changes in virulence and P.D. upon successive passage through white mice of cultures of significantly different virulence and P.D.

II. PRODUCTION OF "ROUGH" STRAINS[2]

The cultures and methods used in these experiments were described in detail in the earlier publications which have been cited. Culture "*A*" is a virulent, type I pneumococcus; cultures "*B*" and "*C*" are variant strains of lesser virulence which were obtained by Professor F. G. Blake from "*A*" by growth in the presence of specific anti-serum and which he kindly placed ar our disposal. The P.D., agglutination and other charac-

[2] We are indebted to Miss Becky Bradley for technical assistance in the course of these experiments.

teristics of these organisms have been described at length by Falk and Jacobson (1925, 1926, 1926a).

Cultures *A*, *B* and *C* were grown in Blake's broth to which had been added 10 per cent of specific antiserum (New York State Department of Health, type I serum, free from preservative). The cultures were incubated at 37°C. and transferred at twenty-four-hour intervals. Control cultures were carried similarly in Blake's broth.

After the fourth transfer, the cultures were plated on sheep's blood agar plates each day and the colonies which developed after sixteen to twenty-four hours' incubation at 37°C. were examined. Definitely "rough" colonies were predominant after the 12th transfers in the broth containing anti-serum. Subcultures were taken from isolated "rough" colonies to blood agar slants. The *A* cultures were carried for 23 transfers; and *B* and *C* strains for 12. Subcultures taken from the 12th and 23rd transfers were used for virulence and other experiments. The notation used to describe our cultures may be illustrated as follows:

A orig. B orig. C orig.	Subcultures taken from stock cultures which had been kept on blood agar slants.
A_{12}, A_{23}, B_{12}, C_{12},	Subcultures taken from smooth colonies obtained by plating out after the 12th or 23rd transfers in Blake's broth + anti-serum.
$A_{12}R$, $A_{12}VR$, $A_{23}R$, etc. $B_{12}R$, $B_{12}VR$, etc. $C_{12}R$, $C_{12}VR$, etc.	Subcultures taken from "rough" colonies obtained by plating out 12th or 23rd transfers in Blake's broth + anti-serum.

Measurements of P.D., virulence for white mice, agglutination and precipitation tests were made by the methods described by Falk, Gussin and Jacobson (1925). Virulence is expressed in M.L.D. (cc.); P.D., in μ/sec. Typical results are illustrated in tables 1 to 4.

From table 1 it appears that smooth colony strains obtained from the 23rd transfer gave virulence and P.D. measurements typical of the original strain. The rough strain ($A_{23}R$) showed

entire lack of virulence for white mice, reduced P.D. and a tendency to spontaneous clumping and sedimentation. After 23 transfers in broth containing anti-serum, both *S* and *R* varieties were still co-existing in the culture. Indeed, in another series of experiments, in which virulence and P.D. were being modified by treatment with pepton in several concentrations, direct observations in the electrophoresis apparatus showed that

TABLE 1

Virulence and P. D. of S and R Strains of Pneumococci.

CULTURE	COLONY FORM	M L D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	7.1
A_{23} (1)	S	10^{-7}–10^{-8}	6.4
A_{23} (1-XX)	S	10^{-7}–10^{-8}	6.7
A_{23} (3)	S	10^{-7}–10^{-8}	6 0
A_{23}R	R	—*	3 2†

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.
† Culture showed spontaneous agglutination and sedimentation.

TABLE 2

Virulence and P. D. of S and R strains of pneumococci

CULTURE	COLONY FORM	M.L.D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	7.1
A_{12} (1)	S	10^{-7}	5.5
A_{12} (3)	S	10^{-7}	7.9
A_{23} (1)	S	10^{-7}	6 5
A_{23} (3)	S	10^{-7}	6.9
A_{12}R	R	—*	5.4
A_{23}R	R	—*	2 6

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.

two varieties of cocci were present—some which moved at high and some at low cataphoretic velocities.

From the data in table 2, it is clear that the findings are entirely similar to those presented in table 1.

In table 3, measurements on the original strains (*A*, *B* and *C*) show the characteristic differences in virulence and P.D. The A_{12} and A_{23} strains, although still showing smooth colonies,

show reduced virulence and reduced P.D. The $B_{12}R_1$ strain was rough but showed no appreciable modification in either virulence or P.D. by comparison with the *B* original strain.

TABLE 3

Virulence and P. D. of S and R strains of pneumococci

CULTURE	COLONY FORM	M.L.D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	8.5
B original	S	10^{-3}	5.0
C original	S	—*	2.9
A_{12}	S	—*	5.0†
A_{23}	S	—*	2.5†
$B_{12}R_1$	R	10^{-3}	5.1
$C_{12}VR_1$	R	0.5	2.5

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.
† Cultures showed spontaneous agglutination and sedimentation.

TABLE 4

P. D., spontaneous agglutination and serum precipitation with S and R strains of pneumococci

CULTURE	COLONY FORM	P.D.	SPONTANEOUS AGGLUTINATION	SERUM PRECIPITATION	
				1 hour	24 hours
		μ/second			
A original (1)	S	7.1	−	++++	++++
A original (2)	S	7.9	−	++++	++++
B original	S	4.0	+	−	++
C original	S	1.9	+	−	++
$A_{12}VR$	R	1.4	+	−	++
$A_{23}VR$	R	1.7	+	−	++
$B_{12}VR$	R	2.9	+++	−	++
$B_{12}VR_1$	R	2.2	+++	−	++
$B_{12}VR_2$	R	2.2	+++	−	++
$C_{12}R_1$	R	1.7	+++	−	++
$C_{12}R_2$	R	1.9	+++	−	++

The $C_{12}VR_1$ strain, although rough in colony form, shows no significant change in either virulence or P.D.

From the data in table 4 it appears that the rough strains of *A* and *C* showed marked reductions in P.D. The reductions for the rough *B* strains were not so great. The tendency of a

culture to show spontaneous agglutination and sedimentation is seen to be an approximate reciprocal of the magnitude of the P.D.

It is significant to note in table 4 that the capacity of the supernatant fluid (obtained by centrifugation of cultures in Blake's broth) to give a precipitin reaction with type I anti-pneumococcus serum within 1 hour (or within the day) is restricted to the *A* original cultures. Neither the variant strains *B* and *C* (Blake) nor the rough varieties produced by 12 or 23 transfers in broth containing anti-serum showed a prompt precipitin reaction. These strains showed a slight but definite precipitin reaction after they were held in the ice box for some twenty-four hours.

III. EFFECTS OF ANIMAL PASSAGE

In earlier publications we pointed out, as Blake and Trask first reported, that when a mouse is killed with the *A* strain of pneumococcus (type I, original) the organisms that are recovered from the heart blood or peritoneal exudate are indistinguishable from the organisms which had been injected. We also confirmed the finding that an occasional mouse injected with a large dose of the *C* strain dies and that from the peritoneal exudate or heart blood only *A* type organisms (of high virulence, high P.D., and low agglutinability) are recovered. Preliminary experiments with the *B* strain, which is intermediate between the *A* and *C* strains in virulence, P.D. and other characteristics, had given variable results. The experiments reported here were undertaken to determine more specifically the behavior of the *A*, *B* and *C* strains upon serial passage through white mice.

Blood agar slant cultures of the original *A*, *B* and *C* strains were covered with a little sterile Blake's broth, incubated over night at 37°C. and the cultures in the broth injected intraperitoneally into healthy white mice. With the *A* strain 0.5 cc. quantities were injected; with the *B* strain 0.75 cc.; and with the *C* strain—which in 0.5 cc. quantity is normally without virulence for mice—1 cc. quantities were injected. The mice died regularly eighteen to thirty-six hours later. A little of the

heart blood was streaked on blood agar plates directly after the animal's death and cultures of pneumococci were recovered from discrete colonies. These were tested with agglutinating serum and with bile and were used subsequently for further injections into mice. A blood agar slant culture of each recovered strain was preserved in the ice chest.

The blood agar slant cultures of the original strains (*A* original, *B* original, *C* original) and of the strains recovered from serial passages through mice (A_1, A_2, etc., B_1, B_2, etc., C_1, C_2, etc.) were used for virulence, P.D. and agglutination tests by the methods which have been described.

TABLE 5

Virulence and P. D. of A, B and C strains of pneumococci after four passages through mice

CULTURE	M.L.D.	P.D.
	cc.	*μ/second*
A original	10^{-7}	7.1
A_4	10^{-7}–10^{-8}	6.8
B original	10^{-2}	5.4
B_4	10^{-3}	3.9
C original	—*	4.1
C_4	10^{-4}†	5.7

* No deaths with 0.5 cc. quantities of undiluted culture.

† Not tested beyond 0.0001 cc. quantity.

The results of the first series of experiments, made after 4 animal passages, are presented in table 5.

The data in table 5 show that both the virulence and P.D. of the *A* and *B* strains were essentially unaltered by 4 passages through mice. The *C* strain had been markedly increased in virulence and perhaps significantly increased in P.D.

The experiment was repeated after 8 animal passages. The results are presented in table 6.

It is evident from table 6 that animal passage has not significantly modified the virulence, P.D. or agglutination reactions of strains *A* and *B*. Strain *C* showed an increase in virulence, an increase in P.D. and a reduction in agglutinability so that, with respect to these characteristics, it is indistinguishable from

strain *A*. It would appear, therefore, that the direct or inverse parallelisms between virulence, P.D., and agglutinability which were discussed in earlier publications are confirmed by measure-

TABLE 6

Virulence and P. D. of A, B and C strains of pneumococci after eight passages through mice

CULTURE	P.D.	M.L.D.	AGGLUTINATION BY TYPE I SERUM
	μ/second	*cc.*	
A original	6.1	10^{-8}	1:40
A_1	6.5		1:40
A_2	6.5		1:40
A_3	6.7		1:40
A_4	8.1		1:40
A_5	4.5		1:40
A_6	5.2		1:40
A_7	6.1		1:40
A_8	6.7	10^{-8}	1:40
B original	5.6	10^{-2}–10^{-3}	1:80
B_1	4.5		1:80
B_2	4.9		1:80
B_3	4 4		1:80
B_4	4.4		1:80
B_5	4 1		1:80
B_6	3.7		1:80
B_7	3 8		1:80
B_8	4.0	10^{-2}	1:80
C original	4.1	—*	1:1280
C_1	4.2		1:160
C_2	5.3		1:30
C_3	5.8		1:20
C_4	5.3		1:20
C_5	5.6		1:20
C_6	(lost)		1:20
C_7	7.2		1:20
C_8	6 0	10^{-8}	1:20

* No deaths with 0.5 cc. quantities of undiluted culture.

ments on cultures subjected to animal passage. The relative constancy of the *B* strain and its failure to revert to the *A* type is a further indication of its comparative stability. This would

appear to confirm the conclusion from the study of single cell strains that the *B* variant is not a mixture of *A* and *C*.

Inasmuch as all three strains have never given detectable "rough" colonies in this set of experiments, it is apparent that strains of pneumococci of different virulence are not necessarily separable into homologous *R* and *S* categories.

After the completion of the series of animal passages which have been described, we undertook two more, similar experiments with the *B* strain. In each experiment, the *B* strain was passed successively through eight mice and the cultures were tested for virulence and P.D. as in the first experiment. The results showed again the greater variability of this strain in virulence and in P.D. as compared with strains *A* and *C*. But they also showed unequivocally in each experiment that after eight passages the characteristics of the *B* strain had not changed significantly towards those of the *A* or the *C* strains.

We have repeatedly observed when conducting electrophoresis measurements that each culture of the *B* strain consists of organisms which, as individuals, give electrophoretic mobilities intermediate between the mobilities of *A* and *C* organisms. The *B* strain is not, from this point of view, a mixture of cocci of the *A* and *C* varieties.

IV. SUMMARY AND CONCLUSIONS

1. The *B* and *C* strains of pneumococci used in these experiments are derivatives of the *A* strain (type I). They were obtained by Professor F. G. Blake by growth in the presence of specific antiserum. The sequence of decreasing virulence for white mice, decreasing P.D. and increasing agglutinability is: *A*, *B*, *C*.

2. Of the colonies of *A*, *B* and *C* strains which form on pepton, serum or blood agar plates, only "smooth" varieties have been observed.

3. Strains which give "rough" colonies on blood agar plates were produced from *A*, *B* and *C* cultures by growth in broth to which specific anti-serum had been added.

4. After 12 or 23 transfers in serum broth, none of the cul-

tures had been completely converted to the *R* varieties. Organisms of both the *S* and *R* varieties could be recovered.

5. Some of the *S* varieties which were recovered after 23 transfers in broth containing anti-serum showed the virulence and P.D. which are characteristic of the original cultures.

6. Some of the *S* varieties recovered showed reduced virulence and P.D.

7. The "rough" varieties recovered after 12 transfers of *B* and *C* strains in broth + anti-serum showed the same virulence and P.D. as the original *B* and *C* cultures.

8. It would appear that strains of pneumococci which differ significantly in virulence are not necessarily separable into homologous *S* and *R* categories.

9. The original *A* strain gives a prompt precipitation reaction with specific anti-serum. The original *B* and *C* strains and the *R* derivatives of *A*, *B* and *C* strains give only a slight, delayed reaction after twenty-four hours.

10. The *A* and *B* strains show no significant changes in virulence, P.D. or agglutination after 4 and 8 passages through mice.

11. Passage of the *C* strain through mice results in a reversion of its characteristics to those of the *A* strain.

12. In all the cases studied, alterations in the virulence of pneumococci for white mice were accompanied by parallel alterations in P.D. and by reciprocal alterations in agglutinability.

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SUGAR AS A SOURCE OF THE ANAEROBES CAUSING EXPLOSION OF CHOCOLATE CANDIES

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In an earlier paper (Weinzirl, 1922) the writer presented evidence showing that the explosion of chocolate candies is due to the fermentation of sugar by anaerobic bacteria. This work has recently received confirmation by Hill (1925). In seeking the source of these anaerobes it seemed logical to assume that some of the materials entering into the fondant served as a vehicle. It was well known that milk and milk products contained anaerobes, hence these might serve as possible sources when incorporated in the fondant. Egg-white is also employed in making fondant, hence samples of commercial egg-white were secured from candy manufacturers and tested for anaerobes. These tests showed that anaerobes were commonly present. The egg-white is doubtless contaminated by hen feces which are known to contain anaerobes. Water also contains anaerobes but usually in very low numbers, hence, it too, may furnish occasional contamination.

In discussing my earlier paper Dr. Nicholas Kopeloff, formerly of the Louisiana Sugar Experiment Station, suggested that the sugar in the fondant might serve as another source of anaerobes. This suggestion was investigated and the present paper presents the results. It will be shown that anaerobes are present in gram samples of practically all kinds of sugar.

METHODS EMPLOYED

Samples of sugar of different kinds were secured from sources widely separated. Some samples were Louisiana cane, some were Hawaiian cane, while others were beet sugar from Colorado and other states.

These sugars were examined by adding 10 grams to 100 cc. of beef bouillon, then transferring 10 cc. of the solution to sterile test tubes, thus making the ultimate sample one gram of sugar. The tubes measured 15 × 150 mm. and contained 0.5 cc. of paraffin. The sugar solution was then heated to 80°C. for ten to fifteen minutes to kill vegetative forms, to permit the paraffin to come to the surface and serve as a seal and to promote anaerobiosis.

The bouillon-sugar cultures were incubated at 37°C. for forty-eight hours to permit multiplication of the anaerobes. If gas was produced, as indicated by the lifting of the paraffin seal, anaerobes were isolated by means of the capillary tube method. From each culture tube showing gas, a series of dilutions was made in tubes containing melted agar. Slender glass tubes (4 × 200 mm.) were filled from each dilution and sealed at both ends. These tubes were then incubated at 37°C. for from two to many days and examined for anaerobic colonies. When colonies appeared the glass was cut and the colonies fished into deep agar tubes and onto agar slopes. If the deep agar stab alone showed growth the culture was known to be an obligate anaerobe. These cultures also served as stock for subsequent study and identification.

RESULTS OBTAINED

By the method outlined six samples of sugar (10 portions from each making 60 grams in all), were tested for anaerobes with the following results:

Table 1 shows that anaerobes were present in 51 of the 60 gram samples, or in 85 per cent of the total number.

The method adopted for detecting the presence of anaerobes is rather crude, hence the results must be regarded as only approximate. It was thought advisable, therefore, to repeat and somewhat refine the work. Instead of gram samples, half gram samples were employed; to the enrichment tubes, brom-thymol blue was added as an indicator of fermentative changes; a small piece of meat was also added to promote anaerobic growth; incubation at 37°C. was extended to seventy-two hours or longer.

Only those tubes which showed gas production were tested for anaerobes. Both the original sugar dilution tubes and the capillary tubes were incubated for anaerobic colonies. About one-half of the samples were also tested by the agar plate method, incubating under anaerobic conditions according to the method of McIntosh and Fildes (1916). Both the deep agar shake culture and the plating methods proved satisfactory, but the capillary tubes were abandoned except for the purification of cultures. In this manner 27 samples of sugar were tested with the results shown in Table 2.

TABLE 1

Showing results of testing sugar for anaerobes

SAMPLE NUMBER	TIMES ANAEROBES WERE FOUND IN GRAM SAMPLES	
	Positive	Negative
1	6	4
2	10	0
3	10	0
4	8	2
5	7	3
6	10	0
Total	51	9

In this series of tests anaerobes were present 115 times in 270 half gram samples, or 42.6 per cent. This is one-half the frequency observed in the first series. Since the samples were one-half as large, it is apparent that the results confirm those of the earlier trial. It appears certain, therefore, that nearly all gram samples of sugar contain one or more anaerobes, and that sugar furnishes a ready source of the anaerobes causing explosion of chocolate candies.

Table 3 is a recalculation of the data given in table 2, on the assumption that gram samples would show anaerobes twice as frequently as one-half gram samples. It is doubtful whether the differences shown are really significant, since the individual tests vary quite widely.

SPECIES OF ANAEROBES FOUND

During the earlier study 56 pure cultures were isolated, and during the later, 28 more. These cultures were studied and their morphological and cultural reactions determined. Five types

TABLE 2

Showing results of testing 27 additional samples of sugar for anaerobes

SAMPLE NUMBER	KIND OF SUGAR	TIMES ANAEROBES WERE FOUND IN 0 5 GRAM SAMPLES	
		Positive	Negative
7	Loaf	2	8
8	Loaf	1	9
9	Loaf	3	7
10	Loaf	4	6
11	White cane	2	8
12	White cane	2	8
13	White cane	3	7
14	White cane	3	7
15	White cane	3	7
16	White cane	5	5
17	White beet	5	5
18	White beet	6	4
19	White beet	5	5
20	Brown	5	5
21	Brown	4	6
22	Brown	6	4
23	Brown	3	7
24	Brown cane	7	3
25	Brown beet	2	8
26	Brown beet	5	5
27	Brown beet	9	1
28	Powdered	9	1
29	Powdered	7	3
30	Powdered	6	4
31	Powdered	4	6
32	Powdered	3	7
33	Brown cane	1	9
27		115	155

were found, two of which did not agree with the descriptions of any species, the others being *Clost. sporogenes*, *Clost. putrificum*, and *Clost. aerofetidum*. *Clost. sporogenes* was the prevailing type found.

TABLE 3

Giving percentage of gram samples showing anaerobes in different kinds of sugar

KIND OF SUGAR	NUMBER OF GRAM SAMPLES	PER CENT FOUND	
		Positive	Negative
Loaf	40	50	50
White cane	60	60	40
White beet	30	100	0
Brown	40	90	10
Brown cane	20	80	20
Brown beet	30	53	47
Powdered	50	100	0

SUMMARY AND CONCLUSIONS

1. Thirty-three samples of sugar of various kinds were analyzed and the relative number of anaerobes present determined.
2. Anaerobes were found in 85 per cent of the 330 gram samples tested.
3. The different kinds of sugar showed about the same number of anaerobes, but the number of samples of each kind was too small for drawing positive conclusions.
4. Five types of organisms were found, as follows: *Clost. sporogenes*, *Clost. putrificum*, *Clost. aerofetidum*, and two not identified.
5. The conclusion is drawn that sugar furnishes a ready source of the anaerobes causing the explosion of chocolate candies.

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IRREGULARITIES IN THE TEST FOR B. COLI IN WATER

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The test for *B. coli*, while considered the most useful method available for determining the sanitary quality of water, is subject to certain irregularities, chief of which is the occurrence of presumptive positive tests which fail to reveal the presence of *B. coli* group organisms when subsequently subjected to the usual confirmatory tests.

These non-confirming presumptive positive tests, which occur with great frequency in certain waters, particularly after chlorination, have been ascribed by various investigators to the presence of aerobic and anaerobic spore-forming lactose-fermenting bacteria and to gas-producing symbiotic groups. Several mediums, notably brilliant green bile, have been experimented with for the inhibition of these interfering organisms and extensive investigations of a similar nature are at present in progress.

In Toronto, positive presumptive tubes which fail to confirm appear to be of at least two distinct types, (1) those probably due to one of the above causes, and (2) those in which colon group bacteria were originally present and lost in the confirmatory procedure. The former, which occur more frequently and constitute over 90 per cent of the presumptive positives from the finally chlorinated water, while consuming a great deal of time and prolonging the period required for interpretation of results, do not affect the reliability of the completed tests, whereas the second type, in which a negative result is recorded for a tube actually positive, may lead to errors of considerable magnitude. It is this latter type which will be discussed in the present paper. The following examples (table 1), selected from results actually obtained in routine examination of raw Lake Ontario water

when heavy pollution was known to exist, illustrate the extent to which the value of the *B. coli* test may be vitiated if the results are interpreted on the basis of Standard Methods of Water Analysis, i.e., that failure to obtain aerobic colonies on solid media inoculated from a positive presumptive tube indicates the absence of bacteria of the colon group.

It can readily be seen from the above results that the indicated pollution as judged by the concentration of *B. coli* would have been 100- to 100,000-fold greater if the loss had not occurred. As previously stated these results were obtained when the water was undoubtedly polluted to a considerable degree, as indicated by other tests, chemical and bacteriological, interpreted in the

TABLE 1

B. coli tests on Lake Ontario water—selected results

The first, second and third plus or minus in each column indicates the result of the presumptive test, confirmatory plate and confirmatory broth respectively.

EXAMPLE NUMBER	100 cc.	10 cc.	1 cc	0 1 cc.	0 01 cc	0 001 cc.	0 0001 cc.
1	+++	+−	+−	+−	+++	+++	−
2	+++	+−	+−	+−	+−	+−	−
3	+−	+−	+−	+−	+++	−	−
4	+−	+−	+−	+−	+−	−	−
5	+++	+−	+−	+−	+++	+++	−
6	+−	+−	+++	+++	+++	−	−
7	+++	+−	+−	+++	+++	−	−
8	+++	+−	+−	+−	+++	+++	−

light of past experience with the same source of supply. In this connection it is of interest to note the observation of Stearn (1923) that the presumptive test is correct in many cases not proven by the confirmatory tests. Hale (1926) also mentions the occurrence of occasional anomalies which indicate that *B. coli* was originally present but was missed in confirmation.

This type of failure usually occurs when the water is badly polluted and appears to be seasonal, inasmuch as the majority of such results are obtained during the period October to April of each year. Precott and Winslow (1915) (p. 108, 118), quoting Winslow and Hunnewell (1902), discuss failures with heavily polluted water which they attribute to overgrowth by sewage

streptococci and other forms present in sewage water. Another explanation of failures under polluted water conditions which appears worthy of consideration is that during the growth of colon bacteria in standard lactose broth a H-ion concentration is produced which is lethal to this group of organisms. This first occurred to the writer following discussion some few years ago with Mr. Frank Hannan, Chemist, Toronto Filtration Plant Laboratories, in which he advanced the theory that the pollution of Lake Ontario water during the late summer months would be considerably greater if the high pH value obtaining at that period of the year had not an inhibitory effect on the propagation of *B. coli*, and that the efficacy of lime treatment in destroying this group of bacteria was probably due to the pH limit of tolerance on the alkaline side being exceeded by this process. Scott and McClure (1924) have shown that such is the case. It is the lower limit of tolerance, of course, which is important in regard to the inhibition of *B. coli* by acid production in carbohydrate-containing media.

A survey of the voluminous literature relating to this group of bacteria provides ample evidence that the life of *B. coli* in carbohydrate media is relatively short and that the pH produced during the growth of the organism is the determining factor. It is this property of *B. coli* that is the basis of the methyl red test of Clark and Lubs (1915; 1917), the glucose content and buffer value of the medium employed being so adjusted that coli cultures elaborate acid until the lethal pH zone (rear pH 5) is attained and remain practically constant, while aerogenes cultures destroy all carbohydrate present before reaching this zone and reversion of reaction occurs, the pH in the two cases becoming further apart as the period of incubation increases. A number of investigators have determined and recorded the final pH values of cultures of colon group organisms, and also the optimum and limiting values for growth.

While it appears to be quite generally known that *B. coli* is inhibited by the acidity formed by its own growth in carbohydrate media, this fact does not seem to have been seriously considered as a factor in the failure of positive presumptive *B. coli*

tests to show the presence of colon bacilli on subsequent examination. That the confirmatory tests should be carried out as soon as possible after fermentation has started in order to avoid loss of *B. coli*, however, has been observed by several investigators and this is a requirement of Standard Methods. Burling and Levine (1918) point out that the confirmatory tests should be performed as quickly as convenient, preferably in twelve to twenty-four hours, and that an incubation period of forty-eight hours in 1 per cent glucose or lactose is detrimental to the successful isolation of *B. coli;* and Hinman (1925), in discussing the use of inhibitory substances in the preliminary enrichment media, states that the chance of losing weak organisms of the colon group is much less with lactose broth, especially if the confirmatory tests have been started as soon as gas formation has been demonstrated and before action of the bacteria has produced an unfavorable acid reaction.

Before describing the experiments carried out brief reference will be made to the composition of the medium employed, which was prepared on the same basis as that used in routine examinations in Toronto and in the study of brilliant green bile previously carried out (Howard and Thompson, 1925). In the procedure recommended in Standard Methods, media of the same composition are used in examining both 10 and 1 cc. quantities of water, 10 cc. of medium being employed in each case, and as a result the composition after dilution with the sample differs considerably. This may not be of importance in regard to the beef extract and pepton content but, as has been shown by Burling and Levine (1918), the lactose content of the mixture of sample and medium is one of the determining factors in the possibility of loss of colon bacilli during preliminary enrichment. It can readily be seen that if 10 and 1 cc. respectively are added to two tubes each containing 10 cc. of a 0.5 per cent solution of lactose the final lactose concentration in the former case will be 0.25 per cent and in the latter approximately 0.45 per cent. This difference has particular significance in the light of the findings of Chambers (1920) that lethal acidity was produced in 0.4 per cent glucose but that continued growth and reversal of reaction occurred in 0.3 per

cent glucose after maximum acidity had been reached. It is evident that serious errors might be introduced in this manner with a medium containing an inhibitory compound such as brilliant green. To eliminate this difficulty, three strengths of lactose broth are employed at Toronto, the composition and amount used being so proportioned that when the desired amount of the water sample has been added the composition of the resulting mixture will be similar in each case. Three quantities of water are employed, namely, 1, 10 and 100 cc., the amount of media per tube being 9, 15 and 50 cc. respectively and the strength ratio 1:1.5:2.7, taking the strength of standard lactose broth as unity. The final composition in each case under these conditions is practically the same as when 10 cc. of standard broth is employed with 1 cc. quantities of water according to the procedure recommended in Standard Methods of Water Analysis.

Assuming that the failure of presumptive positive tests to confirm when the water was known to be polluted was due to the production of a lethal H-ion concentration, experiments were carried out to determine if the death of colon group organisms could be prevented, or at least deferred until the confirmatory isolation as ordinarily performed had been made, by increasing the buffer capacity of the medium by the addition of dipotassium phosphate. The results obtained were most encouraging and indicated that the difficulty could be largely eliminated by the employment of an enrichment medium modified in this manner.

All the tests were carried out on 10 cc. quantities of raw Lake Ontario water, and the medium employed contained 4.5 grams beef extract, 7.5 grams Difco pepton and 7.5 grams lactose per liter. As previously explained, the final composition, after adding 10 cc. of the water sample to the 15 cc. of medium contained in each fermentation tube, was practically the same as that obtaining when 1 cc. quantities are examined according to the standard procedure. The desired amount of buffer salt was added to this medium just prior to tubing, and tests were then carried out in parallel with media of the same composition but without phosphate. It was observed that a flocculent precipitate invariably formed in the buffered medium following sterili-

zation in the autoclave. After adding the water, the tubes were shaken to ensure thorough mixing of the contents. In order to obtain a large number of results in a short period of time five tubes of each medium were inoculated from each sample of water, the two sets of tubes being subjected to exactly the same conditions throughout the test. MacConkey's rebipelagar, described by Houston (1913) (p. 171), was employed for the confirmatory tests, this medium having been regularly employed in the Toronto Laboratories for some years with excellent results.

A few preliminary experiments were conducted with lactose broth of the above strength to which had been added 1 gram of dipotassium phosphate per liter. The results indicated that insufficient buffering capacity was provided by this amount of phosphate, sterile plates being still obtained, although less frequently, and the concentration of buffer salt was therefore increased to 2 grams per liter, corresponding to 1.33 grams per liter of standard strength broth. The results with the latter concentration are shown in tables 2, 3 and 4. Series 1 was carried out on consecutive working days during the spring of 1925, series 2 on several periods of consecutive days during the fall of 1925 and the spring of 1926 and series 3 on selected days in the spring of 1926 when pollution was known to be present. The conditions existing on the days on which the tests comprising series 3 were carried out were such that all positive presumptive tests could be attributed to the presence of colon group organisms, thus eliminating interference from positive tests due to other undefined causes when the water is relatively unpolluted, which cannot readily be distinguished in summarized data from those due to the death of colon bacteria during the enrichment process. A summary of all results is given in table 5.

These results indicate that a considerable loss is apt to occur with preliminary enrichment in lactose broth and that addition of dipotassium phosphate to the medium aids in the elimination of this source of error. As previously stated the occurrence of these failures is seasonal, and the frequency of occurrence varies widely. During the period when series 1 was carried out the loss was particularly marked, while in series 2 it was hardly apparent.

It should be pointed out that the three negative confirmatory plates from the buffered medium in Series 2 were obtained when the water was relatively unpolluted and were probably due to causes other than production of a lethal H-ion concentration. In series 3, carried out on selected days when pollution was known to be present, the loss is again evident but not as marked as in series 1. The two sterile plates obtained with the special medium

TABLE 2

Series 1

	STANDARD BROTH	STANDARD BROTH CONTAINING 0 2 PER CENT K_2HPO_4
Number of tubes inoculated	100	100
Presumptive:		
Positive 24 hours	74	77
Positive 48 hours	6	9
Negative 48 hours	20	14
Confirmatory plates:		
24-hour presumptives: Positive	45	77
Negative	29	0
48-hour presumptives: Positive	1	3
Negative	5	6
Confirmatory broths:		
24-hour presumptives: Positive	45	77
Negative	0	0
48-hour presumptives: Positive	1	2
Negative	0	1
Total number positive confirmed	46	79

in this series were undoubtedly due to the production of lethal acidity notwithstanding the presence of the buffer salt. The organisms present on the days on which these two failures occurred were apparently particularly active in the production of acid as on both occasions the other four plates in the group were very thinly populated and of the five parallel plates from the standard medium only one on each occasion was positive, a solitary colony developing in each instance.

Simply recording the number of positive and negative results does not adequately describe the difference in the plates prepared from the two media. In the majority of instances a much larger number of colonies was obtained from the buffered medium than from the standard broth inoculated from the same sample of water, and when the number from the former was low a portion of the latter were usually sterile. When the water was polluted gas

TABLE 3

Series 2

	STANDARD BROTH	STANDARD BROTH CONTAINING 0.2 PER CENT K_2HPO_4
Number of tubes inoculated	100	100
Presumptives:		
Positive 24 hours	51	53
Positive 48 hours	9	14
Negative 48 hours	40	33
Confirmatory plates:		
24-hour presumptives: Positive	46	50
Negative	5	3
48-hour presumptives: Positive	3	6
Negative	6	8
Confirmatory broths:		
24-hour presumptives: Positive	46	50
Negative	0	0
48-hour presumptives: Positive	3	6
Negative	0	0
Total number positive confirmed	49	56

production in the presumptive tubes was usually greater and less variable in the buffered medium, undoubtedly due to the extension of the period of activity of the organism. In this connection it is of interest to note the observation of Bronfenbrenner and Schlesinger (1918) that the amount of gas produced during the fermentation of lactose by *B. coli* varies inversely with the H-ion concentration, other factors being equal, or directly with the

concentration of buffer. Besson, Ranque and Senez (1919) also discuss the relationship of gas production and acid production by *B. coli* in glucose-containing media, which they believe to be two distinct processes occurring practically coincidently.

The confirmatory plates were prepared by diluting one loopful of the presumptive culture with 7 cc. of sterile distilled water and adding one loopful of the resulting mixture to a tube of melted

TABLE 4

Series 3

	STANDARD BROTH	STANDARD BROTH CONTAINING 0.2 PER CENT K_2HPO_4
Number of tubes inoculated	100	100
Presumptive: Positive 24 hours	100	100
Confirmatory plates: Positive	74	98
Negative	26	2
Confirmatory broths: Positive	71	97
Negative	3	1
Total number positive confirmed	71	97

TABLE 5

Summary of all results

	STANDARD BROTH	STANDARD BROTH CONTAINING 0 2 PER CENT K_2HPO_4
Number of tubes inoculated	300	300
Total number positive confirmed	166	232

rebipelagar, which was then mixed and poured into a sterile Petri dish. This procedure, with practice, yields well-distributed plate cultures from normal presumptive positive tubes, but care is required to avoid overcrowding. A sterile plate obtained under these conditions does not necessarily indicate the absence of colon group organisms in the presumptive tube, but only that the concentration of bacteria is insufficient to give a positive

result at this great dilution. In previous work, presumptive tubes which had given a sterile plate by this method were found by the writer to give well-distributed positive plates when a loopful of the presumptive medium was transferred directly to the agar medium, omitting the dilution with sterile water. To produce such a result there must be a deviation from the number of viable organisms per unit volume normally present in the presumptive medium of the order of 10,000-fold. To overcome such a condition either two suspensions of these widely varying dilutions, and possibly others to avoid sterility on one hand and overcrowding and coalescence on the other, would have to be plated or the presumptive tubes would have to be retained for further examination in event of failure of the first test. Both of these procedures would introduce complications and add to the volume of work, and would, of course, be useless if death of the culture had occurred. There appears to be little doubt that modification of the presumptive medium to defer the rapid destruction of colon group organisms by the acidity produced offers the most satisfactory solution of the problem.

Another irregularity, described in a previous paper (Howard and Thompson, 1925) is evident in the results of series 3, i.e., failure of typical colonies to produce gas when fished into lactose broth as the final step in the confirmatory procedure. This, at first, was also attributed to the production of lethal acidity, but the theory was later disproven as it was found that a typically positive confirmatory plate could be obtained by subculturing the negative confirmatory broth in the usual manner. Failures of this type are not confined to the MacConkey medium, as similar results were obtained when employing eosine methylene blue agar, and the cultures formed typical colonies on Endo medium also. For the purpose of the present study these failures can be disregarded as their occurrence appears to be independent of the preliminary enrichment medium employed.

Other modifications of the preliminary enrichment medium which seemed worthy of consideration were increasing the buffer capacity of the medium by employing a larger percentage of pepton (Müller 1922 and Stearn 1923) and decreasing the

amount of acid produced by further reducing the concentration of lactose. Brief series of comparative experiments were carried out with standard broth modified in each of these particulars. The former proved quite ineffective, lactose broth containing twice (1 per cent) and four times (2 per cent) the prescribed concentration of pepton giving approximately the same number of failures as the standard medium. With regard to the employment of broth containing less than the standard amount (0.5 per cent) of lactose, sufficient experiments have not been carried out to warrant any definite conclusion. A brief series of tests consisting of fifty tubes of standard broth inoculated in parallel with fifty tubes of broth containing one-fifth (0.1 per cent) of the standard concentration of lactose showed that while the failures were eliminated the broth of low lactose content was less sensitive with relatively unpolluted water, and the total number of positive tests confirmed was therefore practically the same. There was also a tendency for the confirmatory plates made from the broth containing 0.1 per cent lactose to be overgrown with alkali-forming organisms. This appears to indicate that the acid formed in the presumptive medium aids in the elimination of other forms present in the water under examination and that it serves a useful function in inhibiting the growth of organisms which would otherwise interfere with the isolation of colon group bacteria. If such is the case it would be undesirable to reduce the lactose content to such an extent that inhibition of these other bacteria would be prevented. Further experiments with intermediate concentrations of lactose and with other buffering agents should be undertaken. It is possible that a medium embracing a combination of these modifications might provide optimum conditions for the enrichment of *B. coli*. Studies along these lines, however, will have to be postponed until the fall of the year, when the failures will probably again become prevalent.

The writer wishes to emphasize the fact that modification of the standard medium to increase its buffering capacity is not suggested as a means of eliminating spurious positive tests due to the presence of aerobic and anaerobic lactose-fermenting spore-formers, symbiotic complexes, etc., and that the prevention of

the type of failure herein discussed would by no means eliminate the desirability of developing a medium which would be toxic to organisms giving rise to false presumptive tests and yet be satisfactory for the enrichment of colon group bacteria. The comparative tests conducted with brilliant green bile indicated that the possibility of loss of typical organisms during the confirmatory procedure was very much less in this medium than in standard lactose broth, probably due to the reduced activity of the bacteria in the presence of the inhibitory compound. The employment of such a medium would probably incidentally eliminate failures due to the production of lethal acidity.

In conclusion the writer wishes gratefully to acknowledge the kindly criticism and advice of Mr. Norman J. Howard, Bacteriologist in Charge of the laboratories in which this work was carried out, and Mr. Frank Hannan, Chemist. The media employed in the investigation were prepared by Mr. Chas. Lepper of the laboratory staff.

SUMMARY

1. Failures of presumptive positive *B. coli* tests of polluted water to show the presence of colon group organisms on subsequent examination are recorded which are believed to be due to the production of a lethal H-ion concentration during preliminary enrichment.

2. It is pointed out that while it is generally known that *B. coli* is inhibited by the acidity formed by its own growth in carbohydrate-containing media, this fact does not seem to have been seriously considered as a factor in the failure of presumptive tests to confirm.

3. Results of comparative tests are given which indicate that failures due to the production of a lethal H-ion concentration may be largely eliminated by increasing the buffering capacity of the presumptive medium by the addition of dipotassium phosphate.

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A LIGHT SWITCH FOR THE MICROSCOPE

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It is usually the custom in describing a new use for the microscope or of some attachment thereto, to be profuse with apologies for taking valuable space in an otherwise crowded Journal, but oftentimes such devices are worthy of at least passing notice.

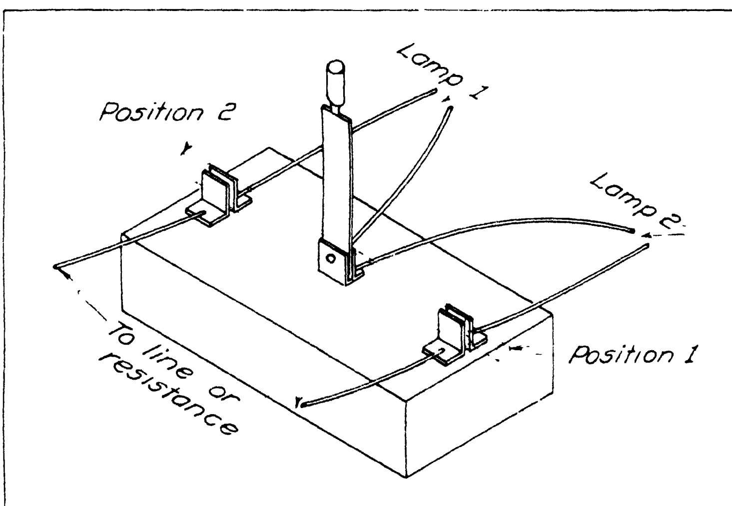

FIG. 1. SHOWING THE PLAN OF THE WIRING

It has been the custom in this laboratory to make use of a dissecting microscope for the study and isolation of colonies on petri dishes. Two sources of illumination were of help in so doing and to avoid a duplication of switches the following hook-up has been found to be of great value.

It is quite remarkable the variety of pictures one can obtain with dual lighting. Our system consists of two lamps placed in series, but with a single-pole double-throw switch across the two lamps. In figure 1 is shown the wiring which may be explained as follows: The current comes to the switch, passes to lamp 1,

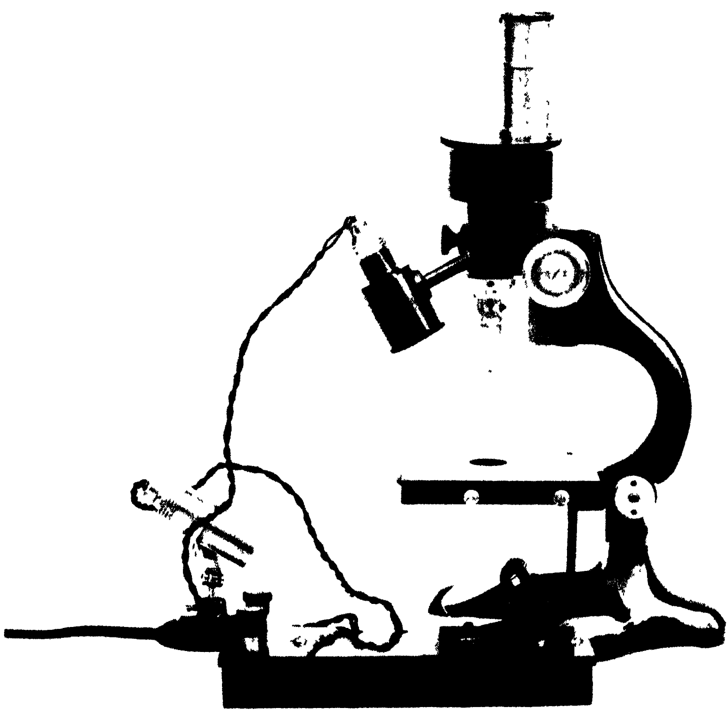

FIG. 2. SHOWING THE TWO LAMPS WITH THE CONTROL SWITCH

Turning the knurled button throws the current into either or both of the lamps

back to the switch, and thence to lamp 2, and out. When the knife is as shown in figure 1, both lamps are on, but when the knife is placed in position 1, as shown in dotted line, then lamp 2 is short circuited, leaving lamp 1 on. The opposite is true when the knife is in position 2.

Figure 2 shows the whole apparatus with the lamps as used.

Perhaps the switch of figure 1 will not be recognized here, since it has been metamorphosed into a compact unit, thanks to the new day of radio and spaghetti tubing.

Figures 3 and 4 show the new switch, which in fact is the same as shown in figure 1. A little study will make clear the arrangement of its wiring.

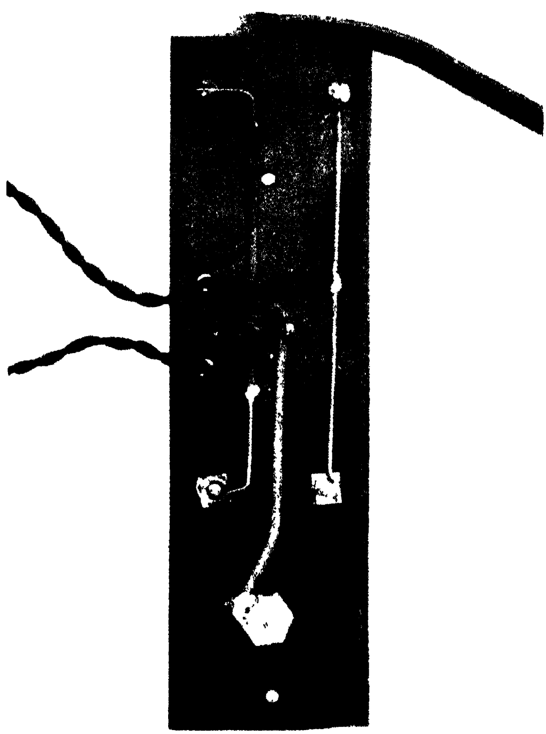

FIG. 3. SHOWING THE SWITCH EXTERIOR IN DETAIL

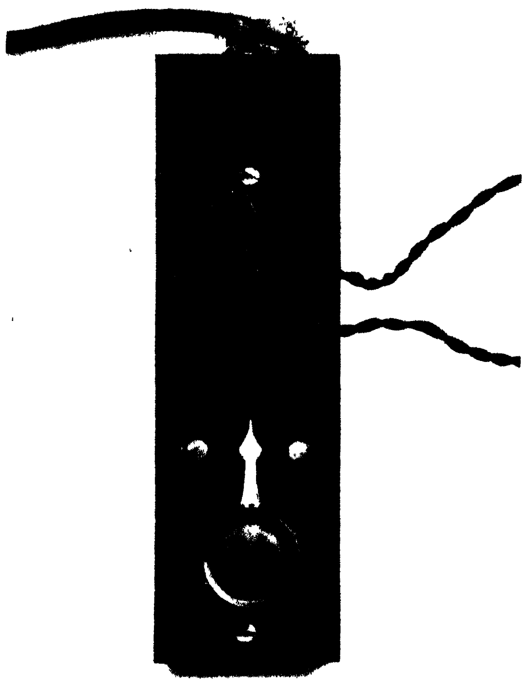

FIG. 4. SHOWING THE INTERNAL WIRING OF SWITCH

ANAEROBIOSIS PRODUCED BY MEANS OF A CENTRAL CATALYZING STATION

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The use of platinized or palladinized asbestos for the combustion of oxygen and hydrogen in an enclosed atmosphere, introduced by Laidlaw (1915), is the basis of modern anaerobic jars. An adaptation of this principle for practical anaerobiosis was first made by McIntosh and Fildes (1916). Later their apparatus was modified by Smillie (1917), and more recently by J. H. Brown (1921; 1922). A large experience with these jars has shown that they should be further improved from the point of view of (*a*) safety, (*b*) rapidity, (*c*) prevention of leakage, and (*d*) multiple use of the same catalyzer. It is our belief that all these advantages have been incorporated in the method to be described.

With respect to safety, the mode of operation includes the use of an almost complete vacuum before the jar is filled with hydrogen. Thus there remains only an infinitesimal amount of oxygen for combination with the hydrogen and hence the possibility of any explosion is prevented. Bursting of the jar was not an infrequent occurrence with prior methods.

In regard to rapidity, the almost complete vacuum again effects a shortening of the time of reaction to from 5 to 10 minutes in place of from thirty minutes to two or three hours, as in the case of the older methods.

Prevention of leakage is obtained by eliminating wires passing through the jar, diminishing rubber tubing to a minimum, and substituting sealed glass connections, and finally by the use of an external catalyzer to replace the older internal, individual catalyzing apparatus.

The greatest advantage of the new method consists in the use of a central catalyzing station, which is placed externally. The station permits many connections, so that any number of jars may be deoxygenated at the same time.

Description of the apparatus.[1] The mechanism has four parts: a central catalyzing station, the jar, the connecting apparatus, and the heating system.

The central catalyzing station.[2] Six or more short, metal connecting tubes are soldered into the outside wall of a flat, cylindrical, metal box, 18 cm. in diameter and 4 cm. high. The palladinized asbestos (5 per cent) is placed in a chamber on the inner surface of the cover and held in place by wire gauze which, in turn, is fixed by screws. On the outer surface of the cover is the heating mechanism. This consists of 1 meter of nichrome wire (0.2 mm. in diameter), wound around a mica plate and insulated by being set between two other mica plates. A sheet of asbestos covers the plates for protection. Over this is screwed a bar of ebonite, into which are inserted two posts with contacts on the under surface, running through the asbestos, for the connection between the house current and the nichrome wire. It is to be noted that the catalyzer can be inspected from time to time, because this cover is merely attached to the lower compartment of the metal box by six screws, with a layer of klingerite interposed. The entire apparatus is supported on a stand which permits elevation and rotation (fig. 1).

The catalyzing mechanism can also be made with glass. (See fig. 2). In this case, the palladinized asbestos is rolled tightly in wire gauze and then fitted snugly in a glass tube of 30 cm. in length and 1.5 cm. in width. The same length of nichrome wire employed in the metal box is rolled around the outer surface of the tube at a level corresponding to the position of the gauze. The wire is attached to two binding posts fitted to the tube by ebonite collars. The tube is sealed at both ends; into it are then

[1] The complete apparatus, including the central catalyzing station, the jar, the connecting system, and the heating mechanism is manufactured by Etablissements Leune, 28 bis, rue du Cardinal Lemoine, Paris, France.

[2] Boëz, L., *Compt. Rend. Soc. Biol.*, 1925, xviii, 1663 and 1666.

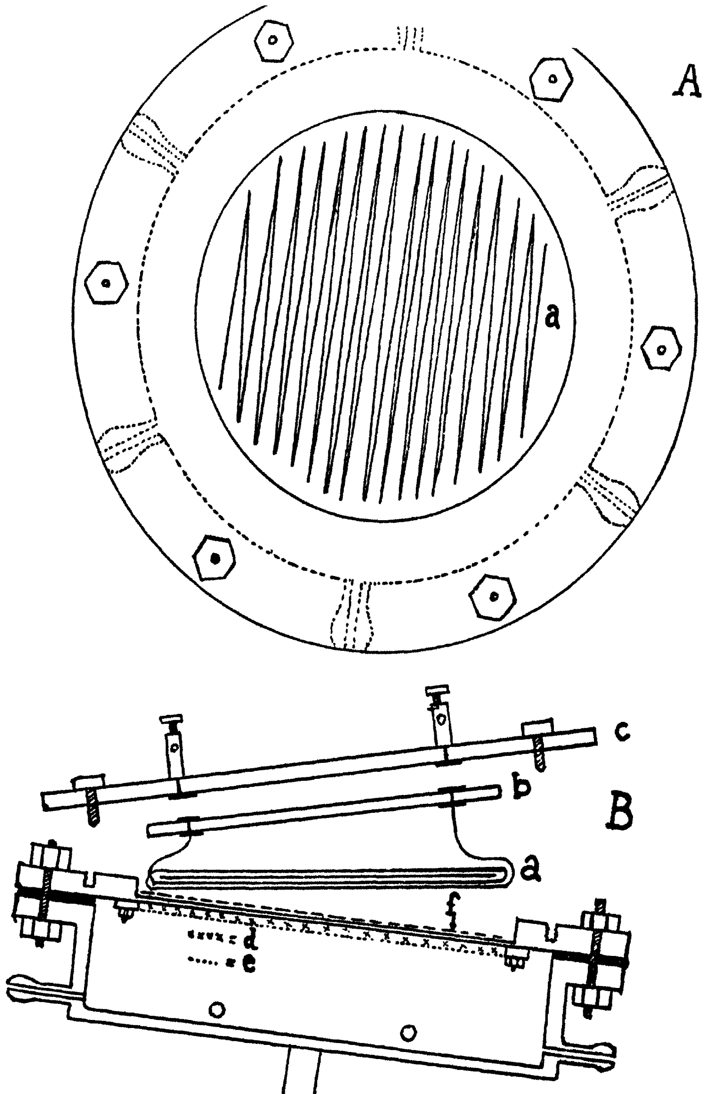

Fig. 1. Central Catalyzing Station in Metal

Schematic drawing: *A*, the upper surface of upper plate, and *B*, a section of the station, with the various parts raised for inspection. *a*, a heating system of nichrome wire; *b*, asbestos layer; *c*, ebonite strip with binding posts; *d*, layer of palladinized asbestos; *e*, wire gauze, and *f*, a mica plate.

fused, at 10 cm. from the upper end, a glass connecting tube, carrying a stop-cock, and at the lower end six or more short glass arms for connecting the catalyzer with the jars. A covering of asbestos cord around the tube completes the structure.

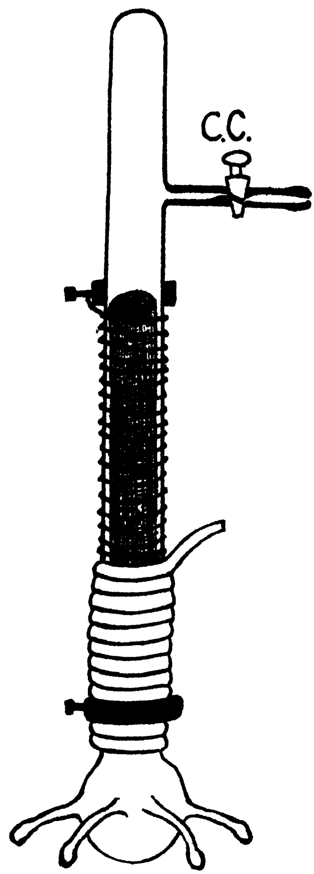

FIG. 2. CENTRAL CATALYZING STATION IN GLASS

Schematic drawing

The jar. This vessel consists of a glass cylinder in the shape of a museum jar, and is made of strong glass, in order to support the vacuum. The upper margin is ground. The top is of copper with the inner surface tinned. Into it is inserted a metal con-

necting tube and this, in turn, carries vacuum rubber tubing, which is sealed at its joint with the metal tube by sealing wax (Golaz' wax is preferable). The rubber tubing is controlled by means of a metal clamp. To close the jar the top is sealed to the cylinder by a good brand of putty. The entire jar is contained in a metal frame, the upper bar of which holds a screw which serves to fasten and tighten the top onto the jar (see fig. 3).

The connecting apparatus. The jar, the catalyzing station, and the hydrogen tank are connected by the following mecha-

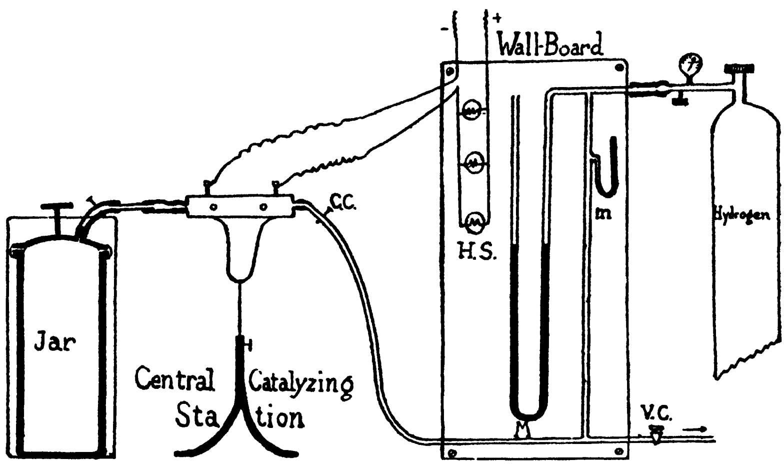

FIG. 3. SCHEMATIC REPRESENTATION OF THE CONNECTING APPARATUS WHICH JOINS THE HYDROGEN TANK, VACUUM, AND HEATING SYSTEMS TO THE CENTRAL CATALYZING STATION AND THE JAR

nism (see fig. 3), which is made of sealed glass tubing throughout and is attached to a wall-board. The apparatus consists essentially of an open arm (*M*) and a closed arm (*m*) mercury manometer. The latter indicates the absolute vacuum reading in the jar and the former measures the rate of inflow of hydrogen. The apparatus is connected at *V* with the source of vacuum; at *T* with the hydrogen tank, and at *C* with the catalyzing station by a rubber tube which is sealed into one of the connecting tubes of the station and carries a metal clamp (*C.C.*) near the catalyzer. At the vacuum arm is placed a tight stop-cock (*V.C.*). To join

the catalyzing post to the jars, the connecting tubes of the station are fitted with short rubber tubes controlled by clamps. By means of glass tubes these are, in turn, connected with the rubber tubes of the jars. It is preferable to seal all joints in this system of connections with wax.

The heating system (see fig. 3, *H.S.*). The heating of the catalyzer is effected by the house current (110 volts), regulated by incandescent lamps, representing an energy of 60 watts. One thus obtains 350°C. in the nichrome wire coil of the catalyzer. This mechanism is affixed to the wall-board.

In addition, the following suggestions are offered to aid in the proper operation of the apparatus. The hydrogen tank should be adjusted with a low pressure valve. The ground glass stopcocks should also be greased with rubber grease[3] and their irregularities smoothed out with a minute amount of lanolin. The rubber tubing is made more impermeable by boiling it in soda solution for several hours, followed by a thorough washing in distilled water. After drying in a current of air, the tubing should be covered with ricin oil on its inner and outer surfaces. Ricin oil has a peculiar property of making all joints impermeable, and is therefore employed to cover all connections and, for the same reason, is also admixed with the putty which is employed to seal the top on the jar.

MODE OF PROCEDURE

The steps in the operation of deoxygenating one or more jars at the same time are as follows:

1. The heating mechanism is turned on.
2. The vacuum connections are opened until a negative pressure of 10–20 mm. Hg is obtained in the jars, as measured by the manometer (*m*). After the vacuum is induced, the cover settles more firmly on the jar; when this occurs the putty is to be remoulded.
3. The vacuum stop-cock (*V.C.*) is shut. Then the manometer (*m*) is watched to detect any possible leak in the apparatus.

[3] To prepare rubber grease, take 2 parts pure rubber, 1 part each paraffin and white petrolatum. Heat for a long time in a water bath at 100°C., until a homogeneous mixture is obtained.

4. The hydrogen tank is opened so that the gas flows at a slow rate. By observing manometer *M* and employing the safety valve on the tank, the rate of flow can be controlled. It is advisable to allow five minutes for the equalization of the pressure. The tank is then shut off.

5. The clamp on the rubber tube connecting the central station to the wall-board apparatus (*C.C.*) is closed.

6. The heat is continued for from five to ten minutes, if one jar is used, and for twenty minutes, if several jars are employed.

7. The clamps on the rubber tubes of the jars are then tightened and the process is completed.[4]

RESULTS

The method reported in this paper has been successful in the cultivation of strict anaerobes of the most sensitive types such as *Bacterium pneumosintes*, *Bacillus ramosus* and *Bacillus fragilis*. *Bacterium pneumosintes*, for example, has often failed to grow in the so-called anaerobic method involving the use of pyrogallic acid and KOH, with or without vacuum, whereas by this procedure cultures always showed a luxuriant and rapid growth. Other anaerobes cultivated or isolated by means of these jars were: *Bacillus welchii*, *Bacillus tertius*, *Bacillus oedematiens*, *Bacillus tetani*, *Bacillus sporogenes*, *Bacillus botulinus*, and many others.

CONCLUSIONS

A safe, rapid, controlled, and effective method is presented for the production of anaerobic conditions for artificial cultures. In addition, by the use of a central, external, catalyzing mechanism more than one jar can be operated at the same time.

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[4] Methylene blue solution is employed as a control of the anaerobic condition within the jars. It is prepared by adding to a test tube containing 5 cc. of sterile 2 per cent glucose, slightly alkaline broth, enough aqueous 1 per cent methylene blue solution to stain the mixture a light blue tint.

THE VIABILITY OF VARIOUS SPECIES OF BACTERIA IN AQUEOUS SUSPENSIONS

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INTRODUCTION

During the past ten years numerous investigations have been reported, from this and other laboratories, in regard to the viability of bacteria in aqueous suspensions. The majority of these studies (Jordan, Russell and Zeit, 1904; Konradi, 1904; Russell and Fuller, 1906; Wheeler, 1906; Whipple and Mayer, 1906; Houston, 1908, 1909, 1911 and 1914; Ruediger, 1911; Clemesha, 1912; Hinds, 1916; Rector and Daube, 1917; Winslow and Cohen, 1918; Winslow and Falk, 1919; Winslow and Falk, 1923 a and b; Meier, 1924; Shaughnessy and Criswell, 1925) have dealt with *Bact. coli* and with forms closely related to *Bact. coli*,—organisms which possess a relatively high degree of viability in water, subsisting in approximately undiminished numbers for twenty-four hours even when suspended in distilled water.

In the course of recent investigations carried out in this laboratory we became interested in the behavior of certain other types of bacteria which appeared to function very differently when suspended in dilute aqueous solutions. The aerobic spore-former, *B. cereus* in particular, seemed to suffer a very rapid mortality in such menstrua and the object of the present study was to investigate this phenomenon and the factors governing it somewhat more closely.

TECHNIQUE

The organisms studied have been four in number, *B. cereus*, *B. megatherium*, *Bact. coli* and *E. prodigiosus*. All were stock

laboratory strains. Cultures were carried on standard nutrient agar and the growth from an ordinary slant was suspended in 100 cc. of sterile distilled water and washed over the surface of standard nutrient agar in a Kolle flask. The Kolle flasks were incubated at 37°C. for eighteen to twenty hours in the case of *Bact. coli* and *E. prodigiosus* and at 20°C. for twelve to fourteen hours in the case of the spore formers, a condition which ensures an abundant development of vegetative cells free from spores. The growth from the flasks was then washed off in 100 cc. of the menstruum to be tested and the suspension filtered through sterile absorbent cotton to remove clumps. Three or

TABLE 1

Viability of B. cereus after centrifuging in distilled water

Bacteria per cubic centimeter

SERIES	PERIOD	pH 6	pH 7	pH 8	pH 9
	minutes				
A	0	12,000,000	171,000,000	242,000,000	271,000,000
	30	13,700	6,600	17,800	9,900
	60	9,000	3,000	12,800	10,200
B	0	7,400	2,500	3,300	14,700
	15	1,600	5,800	1,700	0
	30	0	500	100	100
	60	0	0	0	0

4 cc. of this heavy suspension were then transferred to 100 cc. of the same menstruum to give us the final test suspension and platings were made on agar at appropriate intervals.

VIABILITY IN DISTILLED WATER, WITH AND WITHOUT CENTRIFUGATION

In our first studies *B. cereus* was washed off from the Kolle flasks in distilled water, filtered and diluted as described above, and then centrifuged twice in sterile centrifuge tubes for twenty minutes. The first centrifuging, the first re-suspension and the second centrifuging were performed in ordinary sterile distilled water (slightly acid) in series A and in distilled water adjusted

to pH 7.2 in series B; while the second re-suspension was made in distilled water adjusted to the four different hydrogen-ion concentrations indicated in table 1.

The original number of bacteria present in the centrifuged suspension is indicated opposite the 0 period in the table. Its absolute value has no particular significance since the amount of heavy suspension transferred to the final suspension was not controlled. It is evident, however, that in all instances the bacteria died out very rapidly when suspended in the aqueous menstrua. With the initially high numbers of series A less

TABLE 2

Viability of bacteria suspended in distilled water without centrifuging

Per cent surviving

ORGANISM	PERIOD	pH 6	pH 7	pH 8	pH 9
	minutes				
B. cereus	0	100	100	100	100
	15	79	113	72	58
	45	36	55	281	47
	75	8	160	295	80
	90	104	11	23	13
	120	2	40	23	49
B. megatherium	0	100	100	100	100
	30	24	16	69	11
	60	78	46	86	80
	90	61	132	71	82

than 0.1 per cent of the initial numbers survived at any pH value after thirty minutes while with the small numbers of series B the bacteria had disappeared after one hour.

We next thought it would be of interest to see what would happen if the centrifugal treatment were omitted. In these tests the bacteria were washed off and filtered through cotton as before and then diluted in the distilled water adjusted to the desired pH values. The results in terms of per cent surviving are presented in table 2.

Here we find a wholly different situation. Since the centrifugal treatment has been omitted the 0 period in table 2 cor-

responds to the forty-five-minute period in table 1 and the seventy-five-minute or ninety-minute period in table 2 to the thirty-minute period in table 1,—measuring back to the original washing off of the bacteria. Yet making full allowance for this difference it is evident that in the uncentrifuged suspensions protective substances of some sort were carried over from the agar culture which prevented the sudden mortality which occurs in the centrifuged suspensions. This conclusion was further checked by the tests presented in table 3. In this series all our four test organisms were used and control tests were also made in ordinary nutrient broth,—suspension, filtering, centrifugal treatment and re-suspension being carried out in each case

TABLE 3

Viability in broth and water before and after centrifuging

Per cent surviving

	B CEREUS		B. MEGATHERIUM		E. PRODIGIOSUS		BACT. COLI	
	Broth	Water	Broth	Water	Broth	Water	Broth	Water
Before centrifuging	100	100	100	100	100	100	100	100
After centrifuging	88	0	197	0	44	0	70	280
One hour later	43	0	300	0	37	0	70	131

in the appropriate menstruum (broth or distilled water respectively).

The 0 values in this table do not indicate absolute sterility but in all cases they do indicate that less than 0.01 per cent of the original numbers were surviving.

These data show very clearly that the bacteria studied do not change materially in numbers when centrifuged and suspended in broth for one hour and that *Bact. coli*, as shown by all previous work, maintains itself or even increases in numbers under similar conditions in distilled water. All of the three other organisms tested on the other hand died out almost completely when suspended in distilled water and washed free from protective substances by centrifugal treatment.

EFFECT OF NUTRIENT BROTH UPON VIABILITY

Our next experiments were conducted to determine the concentration of nutrient broth necessary to protect *B. cereus* against the harmful effect of aqueous suspension. Six different dilutions of broth were therefore made in distilled water and these dilute broths were used for washing, centrifuging and re-suspension in the usual manner. The results presented in table 4 indicate that one part of broth in 100 completely protects and one part in 1000 exerts a very considerable favorable influence. It is thus clear why the bacteria survive in uncentrifuged suspensions.

TABLE 4

Viability of B. cereus in dilute broth suspensions

Per cent surviving

PERIOD	BROTH					
	Undiluted	1:10	1:100	1:1000	1:100,000	1:1,000,000
Before centrifuging	100	100	100	100	100	100
After centrifuging	52	37	208	44	0	0
One hour later	52	45	196	12	0	0

VIABILITY IN SALT SOLUTIONS

We next sought to determine the particular factors involved in the protective action of nutrient broth, and first studied the effect of salt concentration with the possibility in mind that osmotic pressure might be the factor involved. Sodium chloride solutions and Ringer-Locke solutions of various strength were used for this purpose. In NaCl of 0.145 M, 0.145 M and 1.450 M strength less than 0.1 per cent of the bacteria (*B. cereus*) survived one hour after centrifuging and in Ringer-Locke solution of double strength, normal strength and one-half strength less than 1 per cent survived at a corresponding period. Clearly the presence of electrolytes will not counteract the harmful effects of suspension in distilled water.

VIABILITY IN SUGAR SOLUTIONS

Although there is of course no sugar entering as a factor into the protective effect of nutrient broth it seemed of some general interest to determine whether the bacteria would survive better in the presence of saccharine substances than in solutions of electrolytes. Tests were therefore made in double isotonic, isotonic and half-isotonic strength of ordinary commercial glucose, but again with wholly negative results. Only one per cent of the organism present survived centrifugal treatment in double isotonic sugar solution and less than 0.5 per cent survived in the lower concentrations.

TABLE 5

Viability of B. cereus in broth, pepton, meat extract and half-strength Ringer-Locke solution

Per cent surviving

	BROTH	PEPTON	MEAT EXTRACT	RINGER-LOCKE
Before centrifuging	100	100	100	100
After centrifuging	56	43	51	3
One hour later	71	53	57	1

VIABILITY IN DILUTE SOLUTIONS OF PEPTON AND MEAT EXTRACT

There remained for consideration as possible protective factors the meat extract and the pepton contained in our nutrient broth. We next proceeded to study the effect of these substances by themselves.

In table 5 are presented data for the viability of *B. cereus* in broth, pepton and meat extract solution and in half-strength Ringer-Locke solution as a control. The broth solution, as usual, contained 5 grams Difco pepton and 3 grams Liebig's meat extract to one liter of water. The pepton solution contained 5 grams of pepton to the liter and the meat extract solution 3 grams of meat extract to the liter. In each case all washing, centrifuging and re-suspension was performed in the particular menstruum studied.

As before the presence of electrolytes (Ringer-Locke solution)

failed to protect; but the bacteria survived in either pepton or meat extract solution about as well as in nutrient broth.

Several series of control tests indicated that *B. cereus* would survive in essentially undiminished numbers in either pepton solution (0.5 per cent) or meat extract solution (0.3 per cent). In a one-hundredth dilution of these same solutions (0.005 per cent pepton and 0.003 per cent meat extract) or in a similar dilution of either pepton or meat extract alone they survive equally well, but in a one-thousandth dilution the protective effect disappears. (See table 6.)

TABLE 6

Viability of B. cereus in pepton, and meat extract solutions

Per cent surviving

	PEPTON		MEAT EXTRACT	
	0 005 per cent	0 0005 per cent	0.003 per cent	0 0003 per cent
Before centrifuging	100	100	100	100
After centrifuging	55	10	62	50
One hour later	59	2	57	0

SUMMARY OF CONCLUSIONS

1. Certain strains of *Bact. coli* survive for several hours in practically undiminished numbers when suspended in distilled water and centrifuged twice to remove substances carried over from the agar slope on which they have previously been grown.

2. The other types of bacteria studied,—*B. cereus*, *B. megatherium*, and *E. prodigiosus*,—when treated in the same way die off almost immediately so that less than one per cent of the organisms originally present are alive one hour after the completion of centrifugal treatment.

3. When the bacteria are suspended in distilled water without centrifuging, the bacteria do not die off in the manner described being protected by substances carried over from the original agar cultures.

4. Salt and sugar solutions are no more favorable than distilled water, to the bacteria studied.

5. On the other hand a menstruum containing one part of nutrient broth in one hundred parts of water completely abolishes the lethal effect and still higher dilutions reduce it materially.

6. The same protective effect is exerted by a similar dilution of either of the two ingredients of nutrient broth (0.005 per cent pepton or 0.003 per cent meat extract) but not by a dilution ten times as great.

7. The influence of meat extract and of pepton in thus promoting the viability of bacteria cannot presumably be due to their use as nutrients since the effect is too rapid and too far-reaching; it cannot be due to osmotic effects since salt and sugar solutions fail to exert any such influence; it would seem probable that the phenomenon is analogous to the operation of a protective colloid.

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SOME FALLACIOUS TENDENCIES IN BACTERIOLOGIC TAXONOMY[1]

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More than any other science, bacteriology suffers for the lack of a satisfactory classification and nomenclature. Yet during the one hundred and fifty-two years since Mueller's first classification of the "Vermes" no less than 66 general classifications, according to Buchanan (1925), have appeared. Prior to Winslow and Roger's (1905–1906) classification of the Coccaceae, the criteria used in the definition and recognition of genera was almost solely morphologic, but these authors introduced two categories of criteria into the definitions of genera that had previously been applied only to the definitions of species, that is, habitat and physiology, and these were soon extended not only to genera but to families and orders by Orla-Jensen (1909). Dobell (1911) also used habitat as a generic criterion in the Spirochaetoidia, and Buchanan (1917) used it as a criterion in defining both the orders and families of bacteria. The views of Winslow and Buchanan, no doubt, dominated the Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (1917) as well as the classification of Castellani and Chalmers (1919), and the Final Report of the Committee (1920). It was most unfortunate that the Final Report was presented only to a small minority of the members of the Society who happened to return from lunch in time to attend a business session of the twenty-first annual meeting, which was held in Boston more than four months before the publication

[1] Read at the twenty-seventh annual meeting of the Society of American Bacteriologists at Madison, Wisconsin, December 29, 1925.

of the report. There was, therefore, no opportunity for scientific consideration (outside the committee) of its contents prior to its adoption, and the approval of the report involved practically no discussion because only a few knew what was coming. The writer was present at this meeting and keenly remembers his own amazement that so important a step as the adoption of "*genera conservanda*" should be undertaken in this way.[2]

Since the "Final" Report there have been two classifications. The first of these, a catalog of new genera of sporulating anaerobes, by Heller (1922), seems to the writer not to deserve extended discussion. The second, an outgrowth of the Society Committee's "Final" Report, was prepared by the new committee and published as Bergey's Manual of Determinative Bacteriology."

I believe that the time has come when we must assess the actual value of these efforts in the light of true, scientific, non-partisan criticism, and in this paper I have attempted to give expression to a train of thought laid nearly seven years ago, and to convictions which have steadily increased during the whole of that period.

The question as to how far up in the scale of classification physiologic criteria should be utilized is one of opinion; I submit that morphologic criteria should enable us to identify the genus and I believe that the definition of orders, families, and genera

[2] Dr. Robert S. Breed raised the point when this paper was read at the twenty-seventh annual meeting that "acceptance" of the report did not involve "adoption" of the genera. In any event it seems to the writer that the force of "acceptance" and "adoption" is exactly the same. In order to record just what action was taken, I secured the minutes of this meeting from the present secretary, Dr. J. M. Sherman. The following is a complete and accurate copy of the references therein to this matter.

"Report of Committee on Characterization and Classification. C.-E. A. Winslow, Chairman, Yale Medical School, New Haven, Conn.

"It was recommended that the names be accepted as definite and approved genera.

"The Committee should get the International Botanical Congress to adopt these as *genera conservanda*.

"It was moved that the Committee on Characterization and Classification be discharged and a Committee on Taxonomy be appointed.

"The report was accepted as read."

should be based solely upon morphologic data. I am willing to admit morphologic properties of the cell that require staining or chemical treatment for their demonstration. On the other hand, I propose to attack the validity of habitat as a criterion in the identification or classification of any living thing. I propose to discuss the desirability of defining species by their cultural and physiologic properties and the futility of attempting to utilize serologic (particularly agglutination) reactions in the definition of bacterial species. I plead for classificatory keys that shall employ clearly defined unambiguous criteria in definitions that shall be mutually exclusive, and a terminology rooted in intimate studies of the *living* organisms by specialists in each field as well as the literature. I believe, most of all, that taxonomic questions cannot be settled by committee action any more than any other scientific question can be so settled, and by this I intend no disparagement of the efforts of the members of the committee, all of whom, I hope, are and will remain good friends of mine. But the task was too great for even these masters of the subject and the time too short for a work which should have occupied not a half-dozen men but a half hundred and not five years but fifty or a hundred and fifty. There is something to be said for usage as a guide, as Shear and Clements (1926) have pointed out.

In Bergey the criteria of the orders are, though probably intended to be morphologic, poorly defined. Who can say what is a "simple" or "undifferentiated" form, or a "specialized" or "differentiated" form? And what is "plant like" or "protozoan like" or "mold like?" Certain properties of these groups overlap as every biologist knows. Thus, we find the latest and most authoritative key to the orders of the class Schizomycetes indefinite and unsatisfactory.

The physiologic definition of the family Nitrobacteriaceae in the order Eubacteriales is almost equally ambiguous and the question may fairly and seriously be raised as to whether any of the four other genera of the Eubacteriales are really excluded. Surely compounds of carbon, hydrogen, and nitrogen are oxidized by organisms in all the families of this order. An otherwise excellent key is spoiled by this.

Turning to the order of Actinomycetales one finds meaningless references to parasitic habitat, and to oxygen and protein requirements in the synopsis of the order as well as in the subjoined key to the families.

Further meaningless definitions are to be found in the synopsis of the order Chlamydobacteriales whose members are described in part as "alga like, typically water forms" "not protozoan like," while the order Spirochaetales is said to be "protozoan like in many characters." What is the use of such expressions?

But it is in the synopsis of the families and the keys to the genera and species that Bergey runs wild in the use of habitat. It appears as a major criterion in the Coccaceae, Spirillaceae, Bacteriaceae, Actinomycetaceae and Spirochaetaceae. Habitat really cannot serve as a criterion in the identification of a bacterium or any other living thing, because it is not intrinsic but extrinsic. If I am found in Africa, does that classify me as a negro? That we use habitat as a guide in the microscopical diagnosis of tuberculosis, gonorrhea, and cerebrospinal meningitis merely happens to be permitted by the peculiar circumstances under which these diseases occur. We are really not any more justified in assuming the *identity* of an organism because it is found in a certain locality than we are in assuming that it is the *cause* of a particular disease merely because it happens to be found in the lesion of that disease, excepting in those few cases where abundant experience has justified such assumptions, and in these cases we constantly run the risk of undetected error. The criteria used in the identification of living forms should be such as to enable one to identify them no matter where they are found and the ubiquity of the bacteria emphasizes the desirability of this need in that group of living things more than in any other.

I do not depreciate the value of information regarding the normal habitat of bacteria; it is indeed most desirable that we should greatly extend our knowledge in this direction. As Hucker (1925) recently pointed out, "a partial correlation between habitat and other characters of the micrococci does exist and this is quite striking in some instances." But we should not attempt to use such data as a basis for identification.

Compared to habitat, the application and limitations of physiologic characters are much more open to discussion. Some physiologic criteria seem to me peculiarly inadaptable to generic distinction. Why should we separate obligately anaerobic spore-bearing bacteria from facultative and obligately aerobic sporulating bacteria, and call one group "Clostridium" and the other "Bacillus"? Aerobes and anaerobes are so closely related that nothing is gained by trying to separate them taxonomically. We do not attempt to separate the anaerobic cocci or the anaerobic actinomyces from the aerobic cocci and aerobic actinomyces. Furthermore several species (*B. histolyticus* and *B. tertius*), described and widely studied as obligate anaerobes, have turned out to be facultative aerobes (Hall, 1923; Hall and Matsumura, 1924), and recent investigations (M'Leod and Gordon, 1925) suggest that we may not be far from the solution of an old bacteriologic problem, that is, the aerobization of the anaerobes. What will become of "Clostridium" then? It is only fair to say that Bergey's use of my name in parenthesis after "Clostridium" was unauthorized and unscientific[3] and his distortion of my key by the insertion of "species," which probably exist only in the literature, entirely misleading.

Our nomenclatural and taxonomic confusion peculiarly involves genera more than it does species and one cannot help noticing the tendency of some of our speakers to omit generic names altogether, using only the specific or even vulgar names of microörganisms, unwilling to accept the new but evidently fearing to retain the old. This is a deplorable situation.

The actual disdain in which the new nomenclature is held by some of those outside the society is indicated in a review of Bergey's Manual by J. L. A. (1924) in Dental Cosmos, in which the critic recalls that Linnaeus placed the entire world of smallest organisms in a single genus, "Chaos," and then recites from Milton,

> Chaos umpire sits,
> And by decision more embroils the fray.

This seems to the writer quite apropos.

[3] Fortunately this was corrected in the second edition.

Witness further this excerpt from Phillips (1925), whose comment appears for the most part unbiased, "to one only a little familiar with bacteriology, the absence of a ready means of finding old friends by index names is not only perplexing, but almost disgusting at unwonted liberties taken." This glaring defect in the first edition has of course been remedied in the second.

That these sentiments are shared by not a few bacteriologists is indicated also in the remarks occasioned by the presentation of Phillips' paper, in which Kendall said, "I do not believe the proposed classification will advance existing and well established procedure very materially. . . . . At the present time, and with existing information, it is difficult to see just how a reasonably permanent classification can be accomplished; that is to say, a classification that shall be obviously satisfactory enough to supplant the present admittedly imperfect system."

And Duval, "Like Dr. Kendall, I do not think we have anything in the new nomenclature that is very much better than what we had before. . . . . I am not inclined to accept the new nomenclature."

And Topley (1926), "Any attempt to adopt such a classification as that proposed by Professor Bergey can only serve in the present state of our knowledge of bacteria, to obstruct any reasonable measure of reform."

Young (1926) has also, in a review of the second edition of Bergey's Manual, called attention to the necessity of considering our newly found appreciation of mutation in connection with classification.

There are others who have similarly expressed themselves to me but I shall not take the liberty of quoting unpublished utterances, given in confidence.

Nomenclature and classification are, of course, two distinct aspects of taxonomy, but they are interdependent and really cannot be separated in discussion. Yet the main problem, as I see it, concerns the definition of the various classificatory groups and the propriety of applying certain differential criteria to them. If we could define orders, families and genera upon morphologic criteria, the logical utility of physiologic criteria would appear

to lie in the identification of species. But wherever physiologic criteria are used there must be definite and clear cut methods for their application, for the metabolic activities of bacteria are influenced by so many factors that have to be carefully controlled, as every one knows, in order to secure comparable results. Yet we must not be too much bound down by "standard methods."

It is difficult to define what we mean by a bacterial "species." We cannot utilize the criterion of fertile interbreeding, for which we may be consoled in the knowledge that it frequently fails as a satisfactory distinction between species among higher plants and animals. But we have an excellent opportunity to place bacteriology upon a most satisfactory taxonomic basis if we can agree to limit physiologic properties to the definition of species, subspecies, and varieties. We may well include pathogenic properties in this category in many cases but there is no reason for basing binomial subdivisions upon serologic agglutination distinctions, as for example, in the genus Streptococcus. It is well known that clearly defined species are subdivided into serologic races by the agglutination and fixation tests as, for example, *Bact. typhosum* (Hooker, 1916), *Diplococcus gonorrheae* (Atkins, 1925), *Diplococcus meningitidis* (Flexner, 1917), *Bacillus tetani* (Fildes, 1925), *Bacillus septicus* (Vibrion septique) (Robertson, 1920), and *Bacillus sporogenes* (Hall and Stark, 1923), to name only a few.

A notable example is *Bacillus Welchii.* Many investigators have attempted and failed to produce serologic agglutinins for it; those few who have succeeded (Simonds, 1915) agree that the serum is strain, not species, specific. This has been my own experience. And yet there are few species of bacteria now so well defined in their essential characters as *Bacillus Welchii.* But if we were to apply a serologic distinction here we should literally have to provide a specific name for each separate strain.

SUMMARY

Bacteriology still suffers for the lack of a satisfactory classification and nomenclature. This is thought to be due to the failure of bacteriologists to agree upon suitable criteria in the

definitions of taxonomic groups. The use of habitat is particularly criticized as inapplicable to the identification of living organisms and the increased tendency to its use in recent taxonomic efforts is deplored. It is suggested that the attempt to settle taxonomic problems by committee action is likely to prove futile.

A plea is made for definitions of bacterial genera and all larger groupings upon exclusively morphologic (including tinctorial) criteria, reserving for the definitions of species physiologic properties, and excluding serologic reactions, particularly agglutination.

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A STUDY IN BACTERIAL MORPHOLOGY

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The great generalization formulated as the cell theory grew entirely out of the work of the botanists. The pioneer work in relation to this theory was already under way in the seventeenth century. The cellular structural pattern of cork and green plants was noted by the botanists Robert Hooke (1665), Malpighi (1675), and Nehemia Grew (1682). The cell nucleus was discovered by Robert Brown, the botanist, in 1831. The cell nucleolus was discovered later, also by a botanist, Gabriel Valentin, in 1836. It was the great botanist, Schleiden (1838), who about this time proved that plant tissue is composed of, and developed from, groups of cells. It was he who gave the anatomist and physiologist, Schwann, the suggestion that led the latter to the observations which enabled him to extend the cell theory also to the animal world. The conception that all living matter, however varied and diverse the gross morphology of individual organisms, is built up from the same type of microscopic unit, gave to biology a simple, uniform working principle of far-reaching influence. The cell theory clearly formulated in Schwann's classic monograph in 1839 opened the way for the extraordinary developments in biological and related sciences that have continued in progressive acceleration since that time. Side by side with the development of botany and animal biology in the seventeenth and eighteenth centuries, the science of bacteriology grew and developed as a corollary of the cell doctrine.

The vision of Kirchner, the Jesuit, and of Lieuwenhoek, the linen-draper, with a passion for perfection in lens grinding, resulted two and a half centuries ago in the revelation of a teeming sub-visible world. The brilliant Abbé Spallanzani

proved, some two generations later, that bacteria, like higher living organisms, can come only from similar preëxisting forms. Upon the foundations established by these and other early workers was built the real science of bacteriology, by Pasteur, the pioneer in preventive inoculation, and by Koch, the developer of the correct theory of specific infectious diseases. It is interesting that of the five makers of bacteriology named above, only one, Koch, was a physician.

On account of the intimate relation of bacteria to human life, and health, and of its economic as well as scientific interest the science of bacteriology is being pursued with great vigor in all parts of the civilized world. Many problems in this field still await solution. One of these unsolved problems concerns the intimate morphology of bacteria. All biologists agree that bacteria are cells, but there is much disagreement as to the internal structure of these cells. Does the bacterium contain nucleus, cytoplasm and cell-wall etc., organized as in the cells which make up higher plants and animals? Upon this question investigators are in sharp disagreement. A critical summary of the views of various authorities has been given recently by Park and Williams (1924) as follows:

1. Bacteria have a definite morphological, more or less centrally situated nucleus (Feinberg, Nakanischi, Shottelius, Swellengrebel and others).

2 Bacteria have no nucleus or differentiated nuclear material (Fischer, Migula, Massert and others).

3. The whole organism, except the membrane which is a delicate layer of cytoplasm, is a nucleus (Bütschli, Löwit, Boni and others).

4. The nuclear material is in the form of chromatin granules distributed throughout the cytoplasm (Hertwig, Schaudinn, Guilliermond, Zettnow and others).

5. A variety of the fourth view is that bacteria possess both the chief elements of a cell, namely, cytoplasm and karyoplasm, but that these are so finely mixed that they cannot be morphologically differentiated (Weigert, Mitrophanow, Gotschlich).

6. Another view advanced, which is a variation of views 3, 4, and 5, is that the bacterial cell is a relatively simple body—a cytode in Haec-

kel's sense, or the plasson of Van Beneden- which possesses both chromatin and plastin, in the relative amounts of these chief substances corresponding more nearly to the amounts found in the nuclei of higher cells than to that of their cytoplasm (Ruzicka, Ambroz).

The last two authors call attention to the fact that both nucleus and cytoplasm in the higher cells are composed of a mixture of chromatin and plastin and that the chief difference between the two mixtures is one of amount and not of kind.

Park and Williams go on to say,

Our studies of bacteria lead us to agree with the views expressed in nos. 4 and 6 of the above summary, that is, bacteria possess both chief elements of a cell, namely chromatin and plastin, and according to the stage of growth and division (varying with the species) the chromatin is in the form of morphological granules or may be so finely divided and mixed with the plastin as to be indistinguishable from it.

Gutstein (1924; 1925), Schumacher (1922), and Laszlo (see Moeller, 1925) in separate recent contributions conclude that the bacterial cell is differentiated into several distinct structures with different chemical characteristics, for which Gutstein gives special terms.

Dobell (1910-1911) stated that all bacteria contain a nucleus, which is variable in form in different bacteria, and at different periods in the life cycle of the same species.

The preceding summary exhibits clearly the utter lack of concurrence among the authorities in regard to the question of the intimate internal structure and organization of bacterial cells.

In this preliminary article we are submitting evidence which bears emphatically upon the question of the internal structure of bacteria. Several micro-photographs are presented which show clearly much of the internal morphology of several varieties of bacteria.

Wright's stain was used for the organisms in all photographs. The lenses used for the photography in connection with this work were as follows:

1. Ordinary high power, which gives an approximate magnifica-

tion of 450. The objective was 4 mm., and the eye piece 10×. (Used in taking first photograph (fig. 1).)

2. Ordinary oil immersion, which magnifies about 1000 times. The objective was 1.9 mm., and the eye piece 10×. (Used in taking second photograph (fig. 2).)

3. Special high power which gave a magnification of about 2300. The objective was of special make, apochromatic in type, 1.3 mm. The eye piece was 25×. The condenser was 12 mm., and aplanatic in type. These three parts were all of

FIG. 1. WRIGHT'S STAIN OF STAPHYLOCOCCUS PYOGENES-AUREUS, MAGNIFIED 450 TIMES, (ORDINARY HIGH POWER) SHOWING CLUSTER FORMATION, BUT NO DETAIL OF INTERNAL STRUCTURE OF THE INDIVIDUAL CELLS

Bausch and Lomb manufacture. (Used in taking all photographs excepting figures 1 and 2.)

Figure 1 is a photograph of a smear of *Staphylococcus pyogenes-aureus*. The tendency of the organism to group in clusters is evident. The individual cells at this magnification, which is that of the ordinary high power, or about 450, are not very clearly discernible. Much less is it possible at this magnification to distinguish their internal morphology.

At the ends and corners of this photograph there appear tiny, somewhat blurred, black circles with light centers. These

are not true representations of the bacteria, but distortions due to the fact that the organisms in these portions of the photograph are out of focus.

There are also a few somewhat similar black circles with light centers scattered over the more central areas of the photograph. These are clear-cut as compared to the blurred figures toward the ends. The clearer, dark rings, with lighter centers, are caused by irremovable particles on the eye piece, as could easily

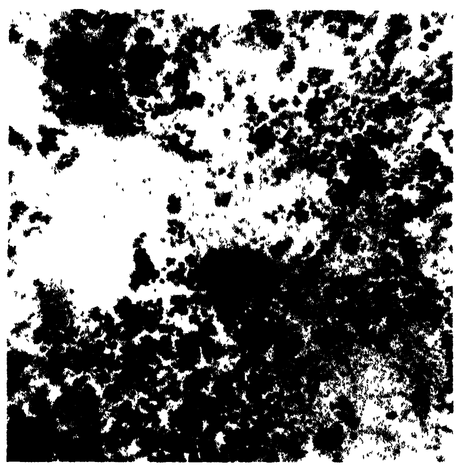


FIG. 2. STAPHYLOCOCCUS PYOGENES-AUREUS WRIGHT'S STAIN, MAGNIFICATION ABOUT 1000 (ORDINARY OIL IMMERSION)

The individual cells are more clearly evident than in figure 1, but there is little intracellular detail evident in the photograph However, some internal structural detail may be seen directly under the microscope at this magnification.

be shown by rotating the eye piece. The masses of staphylococci at the center of the picture which are correctly represented at this magnification appear only as tiny dots, with no internal morphological differentiation apparent.

Figure 2 is a photograph of the same field as was shown in figure 1. The magnification is that of the ordinary oil immersion, or about 1000. The individual cocci are more clearly apparent, yet the internal structural detail of each coccus is scarcely evident. A certain amount of structural detail of the

organism may, however, be made out at this magnification by direct microscopic study.

The reader must not misinterpret the blurred dark rings with the light centers at the periphery of the picture and the few scattered more clearly distinct dark circles with light centers located toward the center. The reason for these has already been explained in figure 1.

The third photograph (fig. 3) tells an entirely different story.

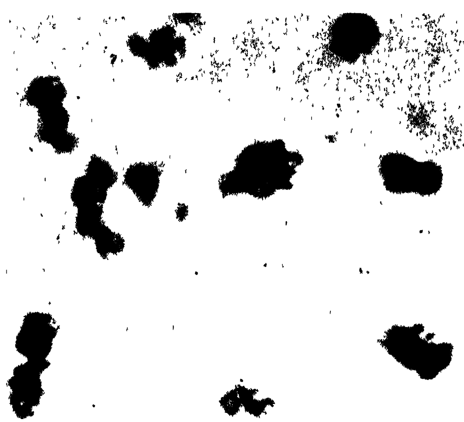


FIG. 3. WRIGHT'S STAIN. STAPHYLOCOCCUS PYOGENES-AUREUS AS MAGNIFIED BY THE BAUSCH AND LOMB APOCHROMATIC LENS SYSTEM. MAGNIFICATION ABOUT 2300

It shows the heavy-staining dense, outer periphery enclosing a light staining, cytoplasm-like area which in turn surrounds a central darkly-staining, small, roundish, nucleus-like spot.

Under the high magnification here employed, about 2300, much of the internal structure of the *Staphylococcus pyogenes-aureus* is clearly evident. Each coccus presents a dark, roundish, nucleus-like center. Surrounding this is a lighter-staining, cytoplasm-like material; and at the periphery is a dense, darkly-staining membrane.

Figure 3a is a diagram which illustrates the plan of structure of the individual coccus of the type shown in figure 3. The peripheral cellwall-like outer membrane is shown at x. The cyto-

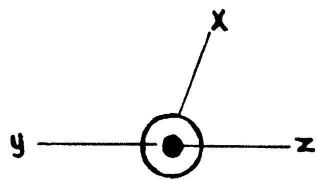
plasm-like body inside the cellwall is labelled *y*. The nucleus-like center is indicated by the reference line *z*.

Figure 3b is a tracing of a group of cocci shown in figure 3.

Figure 3c is a free-hand enlargement of the cluster of staphylococci traced in figure 3b.

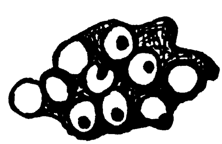
Figures 3, 3a, 3b, and 3c illustrate the general plan of this staphylococcus, which exhibits clearly what appear to be nucleus, cytoplasm and cellwall.

3a



3b

3c



3a. This figure is a diagram illustrating the general plan of these cocci: *x*, cellwall-like periphery; *y*, cytoplasm-like body, *z*, nucleus-like central structure.
3b. Tracing of a cluster of staphylococci seen in figure 3.
3c. Free hand enlargement of the cluster of cocci traced in figure 3b.

The next photograph (fig. 4) of the same organism presents a variation in external shape. Each coccus of the different groups tends to take the form of a polygon, apparently on account of the pressure upon it of neighboring cocci. To this extent, therefore, these organisms when in clusters are more polygonal in outline than they are conventionally described.

Figure 5 is a photograph of the same organism with the same magnification which shows even more clearly the nuclear-like central body in several of the cells. This body stands out especially

FIG 4. STAPHYLOCOCCUS PYOGENES-AUREUS, MAGNIFIED 2300 TIMES. WRIGHT'S STAIN

The polygonal outline of the cocci, which shows well in an upper cluster of this photograph, is due to pressure of these cells upon each other

FIG. 5. STAPHYLOCOCCUS PYOGENES-AUREUS. WRIGHT'S STAIN. MAGNIFICATION 2300, AS IN FIGURES 3 AND 4

Structural plan of coccus identical with that already seen in *Staphylococcus pyogenes-aureus* with same magnification. Note the central nucleus-like, round dark spot, the peripheral membrane, and the intervening lighter, cytoplasm-like area.

well in some of the cells located near the summit of the cluster. Here is shown also very clearly the deeply-staining outer rim of the cocci. The outer dark staining periphery is generally much more clearly evident when seen in the microscope (2300)

than in the picture. The outer wall shows deeper in this picture owing perhaps to the longer time of exposure to the stain.

Figure 6 is a photograph of *Staphylococcus pyogenes-citreus.* The individual organisms of the different groups exhibit the same structural morphology as those already described in figures 3, 4, and 5. In each cell there is a dark, nucleus-like center, a lighter surrounding cytoplasm-like body, and outside of this an outer limiting membrane. The outer membrane is seen much

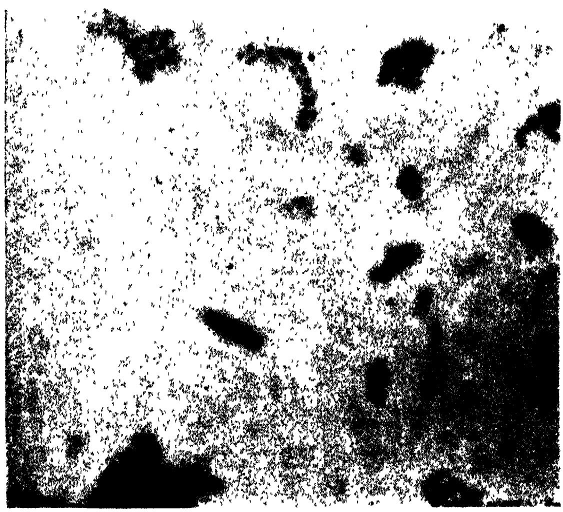


FIG. 6. CLUSTERS OF STAPHYLOCOCCUS PYOGENES-CITREUS. WRIGHT'S STAIN. MAGNIFICATION 2300

Structural plan as in other cocci already studied at same magnification. Note nucleus-like center, outer heavy membrane, and intervening light-colored cytoplasm-like area.

more clearly through the microscope than in the photograph. The *Staphylococcus pyogenes-citreus* therefore exhibits a morphological differentiation similar to that already described for the *Staphylococcus pyogenes-aureus.* The large, heavy, dark areas near the center do not represent bacteria.

In the seventh photograph (fig. 7) is shown *Micrococcus tetragenus.* Several four-cell groups with some larger clusters are here presented. In the individual cells of each group may

be seen a central, small, round, dark nucleus-like spot. At the periphery of the cell is a dark, heavy, cell-wall-like membrane. Between these parts is a lighter-colored cytoplasm-like area, forming the thickest portion of the bacterium. The single cocci of each group present, therefore, a structural plan similar to that of the cocci already described.

Figure 8 is a photograph of a bacillus, the bacillus subtilis. It presents structural characteristics apparently comparable in

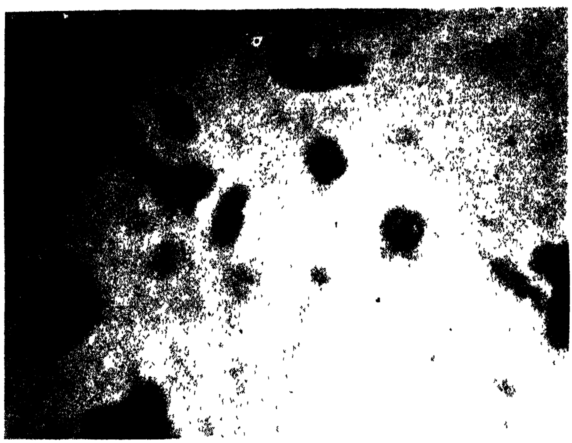


FIG. 7. MICROCOCCUS TETRAGENUS. MAGNIFIED 2300 TIMES. WRIGHT'S STAIN

Several four-cell groups with some larger clusters may here be seen. This coccus presents the same plan of structure as those cocci already studied. In each may be found a central round, dark, nucleus-like dot, and at the periphery a heavy dark, cell-wall-like outer membrane. Between these parts is a thicker, lighter, cytoplasm-like area. The plan of structure of the *Micrococcus tetragenus* is therefore similar to that shown in the cocci already considered.

general to those of the cocci just described. The bacilus shows an outer dark-staining membrane, an inner, axially located, dark-staining, slender, rod-shaped nucleus-like body, and between the outer membrane and the inner slender, axial rod is a more lightly staining, cytoplasm-like structure giving body to the bacillus. The black spots in the photograph are not bacilli.

Figure 8a is a tracing of the bacillus subtilis, shown in fig. 8. The parts which appear to be cellwall, cytoplasm, and nucleus, are marked respectively x, y, and z, as in the cocci in figure 3a.

In recounting the detail of these bacteria we have for descriptive purposes used such terms as nucleus-like, cytoplasm-like, and cell-wall-like for the differentiated portions of the cells on account of the obvious structural similarity of these parts to the

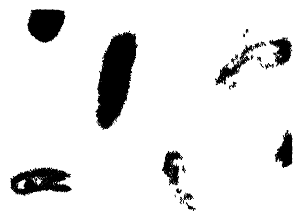


FIG. 8. BACILLUS SUBTILIS. WRIGHT'S STAIN. MAGNIFICATION 2300

This is a photograph of *Bacillus subtilis*. Like the cocci already described, this bacillus presents an outer limiting membrane, an inner, slender, rod-shaped, nucleus like axially located body, and an intervening cytoplasm-like structure. The black spots in the picture are not bacilli.

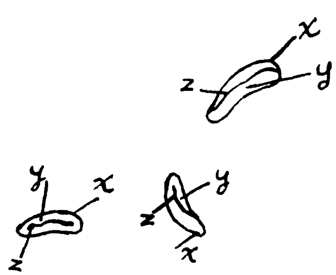


Figure 8a is a tracing of the bacilli in figure 8. The parts which appear to be cell-wall, cytoplasm, and nucleus, are numbered respectively x, y, and z.

In connection with the photograph is a tracing of the same bacillus (fig. 9a). The organism presents intracellular features apparently comparable to those described above for the coccus. There are (x) dark staining periphery enclosing (y) a light staining cytoplasm-like body, and this in turn surrounds (z) the rod-shaped, nucleus-like central structure.

nucleus, cytoplasm, and cell-wall of the cells of higher plants and animals. This structural similarity is evident and striking, yet final conclusions upon the exact identity of the parts so designated with the known parts of the cells which compose higher organisms would be premature at this stage of our investigation.

SUMMARY AND CONCLUSIONS

A study was made of the internal structure of several microörganisms.

1. As seen by the ordinary high-power objective (magnification about 450), no detail of internal structure of the organisms so studied was apparent. Each coccus appeared in the photograph as a tiny round dot.

2. The individual organisms, seen under the ordinary oil immersion (magnification about 1000) stand out more clearly, but their internal structure is little, or not at all, in evidence in the photograph; however, some details of internal structural differentiation can be made out at this magnification directly under the microscope.

3. Several types of staphylococcus and the *Bacillus subtilis* are pictured, with magnification of 2300 in which much detail of the plan of cell structure and organization is clearly evident. It might be said that in general there is for each organism a dark-staining, cell-wall-like periphery, an inner dark-staining nucleus-like, centrally located structure, and an intermediate cytoplasm-like body consisting of more lightly staining material. The central body and the outer membrane of the coccus takes a heavy blue-black shade. The cytoplasm-like material was a bluish-pink. The bacilli took with the same stain a dark color for the central body and the peripheral membrane. These, like the nucleus-like center and cell-wall-like periphery of the coccus, were bluish-black. The cytoplasm-like material of the bacillus stained like that of the coccus a light bluish-pink. The different component parts of the bacilli stained much lighter than those of the cocci.

4. The facts presented in connection with these various microorganisms support the first of the several theories summarized above, namely, that bacterial cells, like those of higher plants and animals are composed of different kinds of structural material, definitely separated from each other in the organization of the cell.

This morphological characterization brings bacteria into

definite structural homology with the cells of higher plants and animals. We are studying other microörganisms, and with varied technique, in an effort to determine the extent of this structural homology in different types of bacteria, and to define more exactly the homology of these structural features with known structures of the cells of higher plants and animals.

We wish to tender thanks to Dr. Francis M. Duffy of the department of Bacteriology for his coöperation in supplying us with bacterial cultures for this study.

We also wish to give sincere thanks to the Bausch and Lomb Optical Company for their interest and assistance in supplying us with the special lenses, apochromatic objective, compensating eye-piece, and aplanatic condenser, which made possible the prosecution of this research. We are especially thankful for the personal assistance of Mr. H. W. Hollister of the Bausch and Lomb Company, who rendered us every courtesy and help in the initiation of our work.

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THE DESTRUCTION OF ACETYL-METHYL-CARBINOL BY MEMBERS OF THE COLON-AEROGENES GROUP

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Various culture reactions and chemical tests have been suggested for differentiating between *Bact. coli* and *Bact. aerogenes.* Some are of merit in differentiating between the fecal and nonfecal strains in water analysis; others are of interest from the standpoint of taxonomy. Since the discovery of the production of the eosin-like color reaction when a strong hydroxide solution is added to certain carbohydrate broth cultures of some of the hemorrhagic septicemia organisms, in 1898, by Voges and Proskauer, and the evidence brought forth by Harden and Walpole (1905–6) that the color reaction was in part due to the formation of acetyl-methyl-carbinol, it has been known as the Voges-Proskauer reaction and has become one of the routine tests for distinguishing *Bact. coli* from *Bact. aerogenes.* It is selected by Bergey (1923) as the differential test for dividing the genus Aerobactor (aerogenes) from the genus Escherichia (coli).

The Voges-Proskauer test is not as simple as its popularity might indicate. One objection to it is the necessity of waiting several hours after adding the hydroxide solution, for the color reaction. Levine, Weldon, and Johnson showed that oxidizing agents would hasten the color reaction but did not recommend the use of any that they tried, for routine work. Another objection to the Voges-Proskauer test is that it does not always correlate with other tests. Linton (1925) substituted a glucose broth for the previously used Clark and Lubs medium for the Voges-Proskauer reaction and found a closer correlation between

the methyl red test and the Voges-Proskauer reaction. He also found that the optimum time for adding the hydroxide solution to the culture was variable. The author (1924) found, while using a glucose-pepton broth (1 per cent of each ingredient) for carrying out the Voges-Proskauer test, that there was a wide variation in the color reaction in cultures of different ages. Some strains of Aerobactor that gave a distinct positive test in thirty-six to forty-eight hours gave a questionable test in four days and a negative test in seven days, consistently. In that investigation tubes were inoculated at twenty-four-hour intervals until there were four of the broth cultures, one, two, three, and four days old. On the fifth day an equal amount of 10 per cent potassium hydroxide solution was added to each tube. In the majority of the cultures the strongest coloration developed in the three- and four-day old cultures, but in a few cases the one- and two-day old cultures gave the most color.

These observations led to the question why the color reaction may not develop in the older cultures of certain strains. Harden and Walpole (1905–6) obtained the acetyl-methyl-carbinol in the distillate, by drying a broth culture of a Voges-Proskauer positive organism under reduced pressure at 55°C. Kling (1905), and others, have found the melting point of acetyl-methyl-carbinol to be 15°C., and the boiling point 148°C. In view of these findings it would not seem probable that the compound is volatilized to any considerable degree at incubation temperature; moreover it would not seem likely to evaporate from some tubes and not from others. Two possibilities remain; it may be destroyed by the organism, or the supply of pepton on which the color reaction partly depends may be exhausted; of these the former seems more probable.

EXPERIMENTAL WORK

None of the cultures used in the previous work by the author were used in this work. Twenty-five of the new cultures were obtained from water, thirteen of which were Voges-Proskauer positive and the rest negative; forty were obtained from renal infections, eight of which were Voges-Proskauer positive and

the rest negative. All the cultures were subcultured on eosin-methylene-blue or Endo plates, or both, and all except two (nos. 5 and 6) were tested at least twice by the methyl-red test and the Voges-Proskauer reaction.

The medium for the methyl-red test was prepared according to the formula given in the Standard Methods of Water Analysis, "Pepton medium for the methyl-red test." After inoculating the tubes they were incubated at 37°C. for four days, then five drops of the indicator were added to each tube and the results recorded at once.

The medium used for the Voges-Proskauer reaction was made of 1 per cent of d-glucose and 1 per cent "Difco" pepton without adjusting; it was tubed in about 5 cc. amounts, and sterilized for fifteen minutes at 15 pounds of steam pressure. The Voges-Proskauer reaction was determined by inoculating a tube of the glucose-pepton broth and incubating it at 37°C. for forty-eight hours, then a second tube was inoculated from the previous one; tubes were inoculated every twenty-four hours thereafter from the previously inoculated tube until 5 tubes were inoculated, thus giving cultures one, two, three, four, five, and seven days old. To these were added equal amounts of a 10 per cent solution of potassium hydroxide; the reactions were observed twelve to fourteen hours afterward and the age giving the most intense color was recorded.

From the 65 cultures were selected three Voges-Proskauer negatives whose methyl-red reaction did not correlate (nos. 15, 22, and 100); eight that were Voges-Proskauer negative and methyl-red positive (nos. 5, 9, 14, 21, 23, 24, 111, and 114); five that were Voges-Proskauer positive and methyl-red negative (nos. 4, 6, 7, 16, and 115) one of which (no. 115) gave a quite transient positive Voges-Proskauer reaction.

Culture number 2, which gave a strong positive Voges-Proskauer reaction in forty-eight hours, was used to prepare a liquid medium containing acetyl-methyl-carbinol. In a 500 cc. Erlenmeyer flask there was sterilized 300 cc. of the glucose-pepton broth which was then inoculated with this strain. After incubating at 37° for forty-eight hours this broth culture was filtered through a Berkefeld filter, then there was added 200 cc. of a sterile 1 per cent pepton

solution, after which the solution was tubed in sterile tubes, about 5 cc. each and incubated for twenty-four hours at 37° to develop possible contaminations. These tubes were then inoculated (except some kept for control tubes) with the various strains of organisms and incubated three days when one tube of each culture together with a control tube was treated with an equal volume of 10 per cent potassium-hydroxide solution and observed twelve

TABLE 1

NUMBER	SOURCE	METHYL RED	VOGES-PROSKAUER		TESTS FOR ACETYL-METHYL-CARBINOL IN ACETYL-METHYL-CARBINOL MEDIUM									
			Test	Optimum time	14 hours	24 hours	36 hours	3 days	4 days	5 days	6 days	7 days	8 days	10 days
				days										
Control														
(2)	Water	−	+	2	±	+	+	+	+	+	+	+	+	+
4	Water	−	+	1–7	+	++	++	±	+	+	+	+	+	+
5	Water	+	−		+	±	+	− −	− −	− −	− −	− −	− −	− −
6	Water	−	+	2–5						±	±	+	+	
7	Water	−	+	4–7				++	+	++	+	++	++	
9	Water	+	−					±	±	+	±	+	+	++
14	Water	+	−					+	+	+	+	±	+	++
15	Water	−	−					±	+	++	+	+	+	++
16	Water	−	+	2–7				±	+	++	+	+	+	
21	Water	+	−						+	+	+	+	++	++
22	Water	−	−					±	+	++	++	++	+	++
23	Water	+	−					++	++	+	+	+	++	+
24	Water	+	−					±	+	++	+	+	++	++
100	Renal	−	−		+	±	tr.	− −	− −	− −	− −	− −	− −	− −
111	Renal	+	−		+	±	±							
114	Renal	+	−		+	±	±							
115	Renal	−	+	2–3	+	±	tr.	− −	− −	− −	− −	− −	− −	− −

to fourteen hours later. Tests were made in this manner on cultures and control tubes that were three, four, five, six, seven, eight, and ten days old, with the results as shown in the table. As cultures no. 5 and no. 100 gave a negative test at the third day when the first test was made and always thereafter, more of the broth containing acetyl-methyl-carbinol was prepared in the same manner and inoculated with these two strains, and

also with nos. 4, 5, 111, and 114, and hydroxide solution was added after fourteen, twenty-four, and thirty-six hours of incubation with the results shown in the table. The very slight coloration in the thirty-six-hour cultures of nos. 100 and 115 bears witness in a striking manner to the earlier negative tests given by these strains with longer incubation periods.

A few words will explain the table. The first column gives the culture number; the second column gives the source; the third column indicates the result of the methyl-red test; the fourth column shows the Voges-Proskauer reaction, and the fifth column shows the age of the Voges-Proskauer positives that showed the strongest color reaction. The reaction given by the control tube (culture no. 2, filtered) is always shown with a (+); when there was a stronger color reaction in the culture tube than that in the control tube it is shown by a (+ +); a weaker coloration in the culture tube than that in the control tube is shown by a (±); when there was only a trace of color in the culture tube it is shown by a (tr.); a negative in the culture tube is shown by a (– –).

While some of the reactions given are not altogether consistent, they are what might be expected when dealing with biological reactions. It is interesting to note that two strains of the Voges-Proskauer negative (nos. 5 and 100) and one of the positive (no. 115) appear to bring about the destruction of the acetyl-methyl-carbinol, and show very constant results.

To gain some evidence as to whether or not the exhaustion of the pepton supply, by the organism, may be responsible for the occasional transient positive reaction, two strains (nos. 19 and 115) which consistently gave transient positive tests, were inoculated in duplicate into tubes of the glucose-pepton medium at forty-eight-hour intervals and incubated at 37°, until four-, six-, and eight-day cultures were obtained. To one series of the duplicate cultures there was added to each tube five drops of a freshly prepared 10 per cent solution of pepton, then each tube was treated with hydroxide solution. In none of the small number of tests made could there be detected any difference in coloration due to the pepton.

CONCLUSION

While more work must be done to furnish conclusive evidence, it seems very probable that the transient positive Voges-Proskauer reaction is due in most cases to the destruction of the acetyl-methyl-carbinol by the organism. There are, apparently, strains of both *Bact. coli* and *Bact. aerogenes* that are able to destroy the compound. A false negative Voges-Proskauer reaction in an old culture of *Bact. aerogenes* is probably not due to the exhaustion of pepton.

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THE SEROLOGICAL CLASSIFICATION OF FUSIFORM BACILLI

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Fusiform bacilli have been known and studied for many years, but relatively little has been done toward classifying these organisms. Several investigators have mentioned the probability that there are several different types. By means of fermentation studies, Krumwiede and Pratt (1913) found two types of fusiform bacilli, one of which fermented sucrose, while the other type did not. Knorr (1922), basing his classification upon morphological characteristics, described three types of fusiform bacilli. No one, however, seems to have studied the serological aspect of this group of bacteria, being deterred possibly by the many obvious difficulties. Broth cultures of fusiform bacilli are impractical for serological work, necessitating the use of surface culture methods for cultivating the organisms. In the past, such methods have been seldom used in the isolation of fusiform bacilli, investigators preferring the shake culture method for this purpose. Knorr was unable to obtain surface cultures of certain of his types of bacilli, even after prolonged cultivation in deep agar tubes to assure acclimatization to artificial mediums. Ellermann (1904), Larson and Barron (1913), Weaver and Tunnicliff (1905), Tunnicliff (1906; 1911; 1923), Mellon, (1919), Krumwiede and Pratt (1913), Brams, Pilot and Davis (1923), and others have isolated and grown these organisms in surface cultures; not, however, in the quantities needed for a serological study. Before such a study could be undertaken, therefore, methods had to be developed both for the rapid cultivation of large amounts of these organisms, and for their rapid isolation from very impure

material; in other words, methods which would enable one to secure surface cultures of known purity, with no possibility of contamination. That such methods are needed may be seen by referring to the literature, in which investigators speak of their "pure" cultures of fusiform bacilli growing aerobically after a few generations; of granules which drop out of fusiform bacilli and give rise to spirilla, and of the transformation of their "pure" fusiform bacilli into motile spirilla, after varying periods of incubation. These observations are sufficient to justify one in the belief that the cultures described by these investigators were impure. As will be shown later, such departures from the typical organism indicate the admixture of other types.

Fusiform bacilli grow in symbiosis with other organisms to a greater extent than has been realized. Some of these contaminating organisms may be so small as to be mistaken for debris, especially when liquid cultures of the organisms are studied. Even in surface cultures, colonies of the contaminating organisms may be too small to be seen with the naked eye. Therefore, since only the surface culture method of isolation, microscopically controlled, offers a means of securing these organisms in a state of unquestioned purity, this method has been used exclusively in the present study. Of course the isolation of a single cell is the most desirable procedure, but it is doubtful if a growth could be secured from an individual cell. The method has not been used in this investigation.

Plaut in 1894 described fusiform-like bacilli found in cases of ulceromembranous angina. Vincent in 1896 described fusiform bacilli and spirilla present in hospital gangrene infections. Bernheim in 1897 reported 30 cases of ulcerative stomatitis and angina in which he found fusiform bacilli in association with spirilla. Vincent in 1898 reported 14 cases of ulceromembranous angina in which these same organisms were present. This infection has since been known as "Vincent's angina."

Veillon and Zuber in 1898 first isolated fusiform bacilli in pure culture. Abel in 1898 succeeded in obtaining the organism in impure culture and kept it alive in this condition for several generations, but was unable to purify his cultures. Lewcowicz

(1903) secured a pure culture of the organism from an infected mouth by isolating it in deep ascitic agar tubes. Pure surface cultures were obtained by Ellermann (1904), who used sodium hydroxide and pyrogallic acid to secure anaerobic conditions.

Fusiform bacilli have frequently been found in lesions about the mouth and throat, usually in association with cocci and spirilla. Weaver and Tunnicliff (1905) and Tunnicliff (1906; 1911; 1923), Krumwiede and Pratt (1913), and others have repeatedly found them in ulceromembranous angina. Matzenauer (1902) Rona (1905), Seiffert (1901), Perthes (1899), Tunnicliff (1911), Krumwiede and Pratt (1913) and others have found them in noma. The two latter investigators have isolated them from carious teeth and pyorrhea. Keilty (1922), while examining the gums of 200 patients, found fusiform bacilli and spirochetes in almost every case. McKinstry (1917) found similar organisms in the mouths of 95 out of 230 healthy recruits during the war. Tunnicliff has observed them in the normal mouth, in diphtheria of the tonsil, and in gingivitis. Brams and Pilot (1923), Tunnicliff (1923) and others have observed them in the normal tonsil. In 1923 the latter investigator isolated a strain of *B. fusiformis* from a normal tonsil, in which she found motile spiral-like organisms. She believed these organisms to be true spirilla, formed in either of two ways: first, by the development of the spirillum from a granule which had fallen out of a fusiform bacillus; secondly, by the rearrangement of the protoplasm within the fusiform bacillus into a spiral-like form, which was then liberated by the breaking down of the cell wall. These observations have not been verified.

Fusiform bacilli in association with various spirochetes and spirilla may cause abscesses in different parts of the body. Lichtwitz and Sabrazes (1899) have observed them in abscesses about the mouth; Schmorl (1907) in a liver abscess; Silberschmidt (1901), Pilot and Davis (1924), Dick and Emge (1914) in brain abscesses. The latter investigators found a brain abscess caused by a pure culture of fusiform bacilli, no cocci or spirilla being present. Pollard (1905) found fusiform bacilli in leg abscesses. Silberschmidt has reported finding them in empyema of the antrum of Highmore.

Pilot and Davis (1924) found fusiform bacilli and spirochetes in pulmonary tuberculosis, lung cavities and bronchiectasis. Rona (1905) found them in two cases of pulmonary gangrene. Greeley (1918), Campbell and Dyas (1917), and others have observed them in bronchitis; Miller (1906) in alveolar abscesses, and McNeill (1924) in an infection of the parenchyma of the lungs, which clinically was indistinguishable from pulmonary tuberculosis.

Müller and Scherber (1904) found these organisms in 50 cases of erosive and 6 cases of gangrenous balanitis, naming the infection "the fourth venereal disease." This disease was previously described by Bataille and Berdal in 1891 under the name of "balano-posthite érosivee circinée." Scherber (1910) later reported 81 cases of this disease. Corbus and Harris (1909), Corbus (1913), Owen and Martin (1916), Brams, Pilot and Davis (1923), Campbell and Dyas (1917), and others have confirmed his findings. Brams, Pilot, and Davis (1913) and Pilot and Kanter (1923; 1924) as well as others have shown that the normal genitalia of the lower classes harbor fusiform bacilli and spirochetes in a large percentage of the cases, both male and female. McConnell (1916) found these organisms in an infection of the cervix.

Generalized infections due to *B. fusiformis* are rare. Larson and Barron (1913) isolated a fusiform-like organism from the blood stream of a patient dying of gangrene. Their description of the organism leads one however to doubt its identity with fusiform bacilli. The same may be said of the "fuso-spirillary" organism isolated by Mellon (1919) from a patient in whom the organism caused a generalized infection, the seat of infection probably being in the appendix.

From the available statistics, it may be seen that fusiform bacilli most frequently attack the mucous membranes. Under special conditions, however, they may attack almost any organ of the body, producing, in some cases, a rapidly advancing necrosis of the tissues, which unless quickly checked, may terminate fatally. It is unfortunate that so few routine anaerobic cultures are made from pathological material, as our knowledge concerning

the distribution of these organisms might otherwise be greatly increased.

ISOLATION AND DESIGNATION OF CULTURES

The data presented in the present paper are based upon a study of 18 pure cultures of fusiform bacilli. In studying this number of cultures, a designation of each strain which would classify it as to its source and morphology was found desirable. Roughly speaking, there are four types of fusiform bacilli which may be separated upon the basis of morphology alone: a narrow, filamentous type; a short, slender type; a broad, stubby type, and one which grows characteristically in long chains, forming wavy filaments which might be mistaken for spirilla. Accordingly, the following system of nomenclature was devised. Cultures from normal areas or from lesions were designated by the letters N or L, respectively. Following these letters were placed letters designating the characteristic morphology of the organism; F for the filamentous type, S for the slender type, B for the broad type, and W for the wavy, spiral type of organism. Similar cultures isolated from different sources were separated by placing Arabic numerals after the type letters.

The following list shows the source of each of the cultures studied:

LF1. Carcinoma of the tongue
LB1. Ulcerated tonsil. Vincent's angina
LB2. Carious tooth
LW1. Tonsillar granule, from excised tonsil
LW2. Excised tonsil
NW1. Tartar from teeth
LS1. Same source as LW1
LS3. Excised tonsil
LS4. Excised tonsil
LS5. Carious tooth
LS6. Excised tonsil
NS1. Normal tooth
NS2. Same source as NW1
NS8. Tooth with very small cavity

NS10. Normal gingiva, extremely dirty
NS12. Tartar
NS13. Tartar
NS14. Tartar

The organisms were isolated by means of surface culture methods only. Undiluted pus, tartar or mucus from the infected area was streaked over the surface of blood agar plates, by means of a special apparatus, devised solely for this purpose, to which was given the name "inoculating machine." By its aid, the plates were streaked in a series of concentric circles, and a far better separation of the colonies on a plate was possible than by hand streaking. By the aid of this procedure the isolation of fusiform bacilli has become a simple matter. A description of the apparatus will be given elsewhere.

INCUBATION OF CULTURES

The inoculated plates were incubated exclusively in anaerobic jars. The method used was that described by the author (Varney, 1926), in which anaerobiosis is secured by burning phosphorus within a tightly sealed glass chamber. A metal rack is constructed, so as to hold the petri dishes. This fits into a standard sized museum jar, and on top of the plates is placed a container for holding the phosphorus. A little water is placed in the bottom of the jar, the phosphorus placed within its container, and the jar tightly sealed. The phosphorus soon takes fire, and quickly establishes anaerobic conditions. Suitable guards are provided to overcome the danger of breakage of the jar.

In practice, after the jar was loaded, it was placed in the incubator and incubated for forty-eight hours. At the expiration of this period, the jar was opened, the phosphorus container immediately removed to the hood, or flooded with water, and the plates removed for examination.

The high moisture content within the jar during incubation greatly aids the growth of fusiform bacilli, and a much heavier growth is obtained than with any other of the common anaerobic methods. Due to the high moisture content, some liquid may

find its way into the bottom of the inverted plates during incubation. If present, this moisture should be removed before an examination is attempted, by pressing the opened, inverted plate down upon a piece of dry filter paper. The plates may then be safely examined.

EXAMINATION OF PLATES

The characteristic morphology of colonies of fusiform bacilli cannot be easily seen when they are examined under a monocular microscope. A dissecting microscope, fitted with a special base, was used for the examination of plate colonies. Instead of using the regular stage, much better results are obtained if a stage is constructed, at an angle of 10° from the horizontal. Plates are placed on this stage, and the colonies viewed by reflected, rather than by transmitted light. Seen in this manner, they are very characteristic, and are distinguishable from colonies of other organisms. When a typical colony is found, a portion of it is picked, by the aid of a sharply pointed needle and transferred to a slide, where it is stained and examined. If typical fusiform bacilli are found, free from other organisms, the remainder of the colony is picked and streaked over the surface of a fresh blood agar plate, which is then incubated for forty-eight hours in the anaerobic jar. If upon examining this plate, none but fusiform colonies are found, it is usually safe to assume that the culture is pure. If but a single contaminating colony is found, however, a new colony should be picked, and the process repeated.

Before the possibility of a mixed growth of fusiform bacilli and other organisms can be definitely ruled out, a well streaked plate culture must be incubated for not less than 6 days, then very carefully examined under the binocular for the presence of contaminating colonies. By this means it is often possible to detect extremely small colonies, from 0.025 to 0.0125 mm. in diameter, growing in supposedly pure cultures of fusiform bacilli, whereas a stained smear from the plate, or a naked eye examination, would show no impurity. In this work, it was found that when a culture gave off a very foul odor, such as has been re-

peatedly mentioned in the literature, it was invariably an indication of the impurity of the culture. Usually in these cases, the contamination was due to bacteria which grew in the extremely small colonies already mentioned. Pure cultures of fusiform bacilli do not give off a foul odor. It is important to know that fusiform bacilli may be contaminated with bacilli, which, by reason of their small size or minuteness of colony, are not detectable by an ordinary examination of a supposedly pure culture.

CULTURE MEDIA USED

When grown on dissimilar lots of media, fusiform bacilli show remarkable changes of shape, hence every precaution should be observed to keep lots of media as nearly uniform as possible. Even on similar lots of media, some types of fusiform bacilli show wide variations in size in different generations. Most fusiform bacilli, however, retain their characteristic morphology, even after many generations, when grown on identical lots of media, but lose it immediately when placed on a medium unlike that to which they have become accustomed. This is shown in figures 9 and 10. Figure 9 shows the normal form of the organism, grown on a medium to which it had become accustomed. The same generation of the same culture, when grown on a medium containing less blood, grew in the form shown in figure 10. If the characteristic morphology is to be retained, therefore, even these slight differences in successive lots of media must be obviated.

Blood agar is the best medium on which to grow surface cultures of fusiform bacilli. The agar base of the medium used in this work is composed of proteose pepton 1 per cent, Liebig's beef extract 0.3 per cent, sodium chloride C.P. 0.5 per cent, and washed, dried agar-agar 1.7 per cent in distilled water. Tap water should never be used. The ingredients should be melted with as little heating as possible, and the reaction very carefully checked before the final sterilization. The final reaction should be pH 7.4. The agar is flasked in carefully measured amounts, so that the proper amount of blood may be added later. Just before use, a flask of agar is melted, cooled to 50°C., and exactly

4 per cent citrated blood added. After thorough mixing, plates are poured or the medium tubed. In this laboratory, large batches of the agar base are made at one time, for the sake of uniformity, and stored in the ice box.

If human beings are used as a source of blood, certain precautions must be observed if successful results are to be obtained. Human blood, secured from clinic patients, has been used exclusively in this work. From time to time certain lots of media failed to support a growth of fusiform bacilli, or but one to two colonies developed, even after long incubation, following a heavy inoculation of the medium with a vigorous culture. Before the source of the trouble was found, some dozen cultures were lost as a result of their failure to grow on this medium. It was finally discovered that the trouble lay in using blood from persons undergoing treatment for syphilis. This blood may contain enough arsenic to inhibit the growth of fusiform bacilli, or so weaken them that sub-cultures cannot be obtained. Other bacteria, with the exception of spirilla, will grow readily on such media. For routine cultivation of fusiform bacilli, therefor, care must be taken to exclude from the medium blood containing arsenic, if successful results are to be obtained.

Under certain circumstances the presence of arsenic in the medium may be of advantage, however. It may be that a great deal of the confusion which has arisen over the relationship between fusiform bacilli and spirilla has been due to reports based on the study of impure cultures, mixtures of these two organisms. In ruling out all possibility of a symbiotic growth between these two organisms, advantage may be taken of the fact that spirilla are somewhat less resistant to the action of arsenic compounds than are fusiform bacilli. If the culture is grown for one or two generations on media containing enough arsenic barely to permit the fusiform bacilli to grow, all spirilla will be killed.

These phenomena have been well illustrated in the present work. Before using arsenic free media exclusively for isolation purposes, no spiral organisms were ever encountered in any of the cultures examined, though thousands of slides were made from cultures one to 355 days old. Frequently "shadow forms"

were seen: old, degenerate cells from which most of the protoplasm had escaped, leaving merely a light staining shell, but no spiral forms were ever seen. Nor have they been seen in these same cultures grown for many generations on arsenic free media.

In later cultures, however, which were isolated and grown on arsenic free media, both wavy types of fusiform bacilli and true spirilla have been found and grown in pure culture. From one supposedly pure culture of fusiform bacilli, growing on a plate, a true spiral organism was isolated after ten days incubation. Transferred repeatedly to arsenic media, it failed to grow, though in each case a growth of fusiform bacilli was obtained. Accordingly, in this laboratory it has been made a practice to cultivate all strains of fusiform bacilli on arsenic blood agar for several generations, in order to rule out the possible presence of a spiral organism.

From these observations, one is justified in the belief that the possibility of a symbiotic growth between fusiform bacilli and spirilla has not been ruled out in those cultures in which motile spirilla have been reported. While the confusion may have arisen through mistaking the wavy type of fusiform bacillus for spirilla, this seems improbable, since there is little real resemblance between the two organisms. It is possible, however, that smears made from liquid or semi-solid cultures of the wavy type of fusiform bacillus could not be differentiated from true spirilla, though no motility is present in the former. The two organisms may be readily differentiated when grown on surface cultures, however.

Experiments have been planned to ascertain the exact quantity of various arsenic salts needed to inhibit the growth of spirilla, while still permitting the growth of fusiform bacilli. At the present time no recommendation can be given as to the exact quantity needed.

STOCK CULTURES

Stocks of all bacilli isolated have been kept in autoclaved brain medium under vaseline, in a modified Eberson's yeast medium, and on the surface of blood agar slants under pyrogallic acid.

Pure cultures of fusiform bacilli grow poorly in Eberson's medium, but in mixed cultures, preferably with streptococci, they grow readily. Brain cultures remain viable for several months, whereas slant cultures on blood agar should be transferred every ten to twelve days in early generations.

IMMUNIZATION OF ANIMALS

Beginning with the tenth to the fifteenth transfer of the pure cultures, rabbits were immunized by the intravenous injection of living forty-eight-hour cultures of LFl, NS13, NS14, and LS5, all of which showed morphological differences when examined under the microscope.

Two methods were used in preparing the antigens. In the first method, the organisms were grown on blood agar slants, incubated in the anaerobic jar for forty-eight hours, then washed off with sterile saline. In the second and preferable method, the organisms were streaked over the surface of blood agar plates by means of the inoculating machine, incubated for forty-eight hours in the anaerobic jar, then washed off with saline. Too heavy an inoculation decreases rather than increases the amount of growth. A well growing plate culture of fusiform bacilli should furnish from 6 to 10 cc. of antigen. Cultures incubated by Wright's method furnish too small a volume of organisms for injection or agglutination purposes.

A few strains of fusiform bacilli form granular suspensions, due to the tendency of the organisms to remain in clumps, or to agglutinate spontaneously. This does no harm when the suspension is to be used for injection, and the antigen need not be shaken before injection. Due to this clumping, however, it is impossible to count the number of bacteria to be injected accurately, hence the injections should be governed either by the number of cubic centimeters of a standard suspension used, or by the number of plate cultures used. Since the amount of growth on plates varies, it is more accurate to inject a definite volume of the suspension each time. The initial volume used is 1 cc., which is increased by 1 cc. at each subsequent injection.

Intravenous injections only were made, with forty-eight hour

intervals between injections. With one exception, no reactions have been observed. In the exception noted, the respiration rate was markedly increased for a period of thirty minutes. Any number of injections may be made without harm to the animals.

The titre of the serum was tested after the fifth to the ninth injection. If this was over 1:5000, which was considered satisfactory, the animal was then bled. Bleeding was first practiced at an interval of ten days after the last injection, but this has been found to be entirely too long an interval, the titre dropping abruptly within this period. The serum of one animal, injected nine times, had a titre of 1:81,920 at the trial bleeding, which was performed two days after the last injection. At the end of nine days, when the animal was exsanguinated, the titre had dropped to 1:20,480. Starin and Dack (1923), studying the immunological response elicited in rabbits immunized against *Clostridium botulinum*, found that the titre of their serum rose steadily for seven to eight days after the last injection, after which it fell. They recommended bleeding five to seven days after the last injection. Other investigators, working with animals immunized against different anaerobes, have found that the titre begins to drop rapidly three days after the last injection, confirming in this respect the results obtained in the present investigation. Accordingly, animals immunized against fusiform bacilli are now bled three days after the last injection.

Blood was drawn aseptically from the carotid artery, and the serum preserved by the addition of 0.1 cc. of 5 per cent phenol in saline to each cubic centimeter of serum collected. The titre was not affected by the preservative.

PREPARATION OF ANTIGENS USED IN THE AGGLUTINATION TESTS

Antigens used for agglutination purposes were prepared in a manner similar to those used for injection, with the exception that those cultures which formed granular suspensions were thoroughly shaken for five or ten minutes to break up all clumps. Too long a shaking should be avoided. Even after this treatment, it was extremely difficult to keep some of the antigens in even suspension during the tests. With some of the strains used, as with other

anaerobes, the tendency toward spontaneous agglutination was very great. The control tube is absolutely essential in all tests, to detect any trace of spontaneous agglutination.

Cultures from twenty-four to 48 hours old are essential for successful results. Old cultures used for preparing antigens usually agglutinate spontaneously. Antigens or cultures which have remained in contact with the air for some time are worthless for agglutination purposes. The more readily a culture auto-agglutinates, the younger the culture should be which is used to prepare the antigen.

Absorption tests probably would be more suitable for typing fusiform bacilli than agglutination tests, but these have not been attempted.

THE TEST

In order to conserve antigen, a preliminary test of each organism was made, with dilution of 1:1 to 1:20. After incubating at 40°C. for ten minutes, the result could usually be read. Agglutination often occurs in five minutes in these low dilutions. In several cases, agglutination with more than one serum occurred, showing the presence of sub-types. In no case did sub-types agglutinate in dilutions greater than 1:20. In dilutions greater than this the reaction is very specific.

Having found the probable type to which an organism belonged by means of this rough test, a series of dilutions of the serum were prepared, ranging from 1:20 to 1:20,480, placing 0.5 cc. of each dilution in a tube. A like amount of antigen was then added to each of the tubes, which, after a thorough shaking, were placed in the water bath at 40°C.

Agglutination often occurred in five or ten minutes in the highest dilutions employed. A period of two hours was allowed as the maximum. In an effort to obviate spontaneous agglutination various temperatures of incubation were tried, with negative results. A temperature of 45°C. is apparently less efficient than one of 40°C., while incubation at 56°C. cannot be practiced, since the bacteria immediately agglutinate and rise to the surface of the liquid. The bacteria settle out rapidly at very low tem-

TABLE 1

Types of fusiform bacilli

TYPE I	TYPE II	TYPE III	TYPE IV
LF1	NS14	LW1	LB1
Sub-type 1	LS4	LW2	LB2
LS5	LS6	NW1	
LS3			
LS1			
NS2			
NS10			
Sub-type 2			
NS1			
NS8			
NS13			

TABLE 2

Agglutination reactions with Type I serum

ANTIGEN	1:1	1:8	1:16	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	CONTROL	TEMPERATURE	TIME
															°C.	
LF1	+	+	+	+	+	+	+	+	+	+	+	+	+	–	40	15 minutes
LW1	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	24 hours
LW2	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	24 hours
NW1	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
LS1	+	+	±	±	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS3	+	+	+	–	–	–	–	–	–	–	–	–	0	–	40	2 hours
LS4	±	–	–	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
LS5	+	+	±	±	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS6	–	0	0	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
NS1	±	+	+	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
NS2	+	+	+	±	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS8	+	+	+	+	±	±	–	–	–	–	–	0	0	–	40	2 hours
NS10	+	+	+	±	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS12	+	+	+	±	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS13	+	±	±	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS14	–	0	0	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS5	+	+	+	+	+	+	+	+	+	+	0	0	0	+	56	5 minutes
NS1	+	–	–	–	–	–	–	–	–	–	0	0	0	–	45	22 hours

peratures, hence results cannot be read if the tubes are placed in the ice box. Hall and Stark (1923) suggest that this settling out

of anaerobes when placed in the ice box may be due to a negative chemotactic response to oxygen. Fusiform bacilli, however, are more sensitive to cold than to oxygen, settling out immediately after chilling, whereas they will remain in contact with oxygen for several hours without settling out, if kept at 40°C.

A rapid decrease in the titre has been observed in all four sera, confirming observations made a year previously, when four

TABLE 3

Agglutination reactions with Type I, Sub-type 1 serum

ANTIGEN	1:1	1:8	1:16	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	CONTROL	TEMPERATURE	TIME
															°C.	
LF1	+	+	+	+	±	±	−	−	−	−	−	0	0	−	40	2 hours
LW1	−	−	−	−	−	−	−	−	0	0	0	0	0	−	40	2 hours
LW2	−	−	−	−	−	−	−	−	0	0	0	0	0	−	40	2 hours
NW1	−	−	−	−	−	−	−	−	0	0	0	0	0	−	40	2 hours
LS1	+	+	+	+	+	+	+	+	+	±	±	0	0	−	40	5 minutes
LS3	+	+	+	+	+	+	+	+	+	±	−	0	0	−	40	5 minutes
LS4	−	−	−	−	−	−	−	−	−	−	−	0	0	−	40	2 hours
LS5	+	+	+	+	+	+	+	+	+	+	+	+	0	−	40	5 minutes
LS6	−	−	−	−	−	−	−	−	−	−	−	0	0	−	40	2 hours
NS1	±	±	−	−	−	−	−	−	−	−	−	0	0	−	40	2 hours
NS2	+	+	+	+	+	+	+	+	+	+	−	0	0	−	40	15 minutes
NS8	+	±	±	−	−	−	−	−	−	−	−	0	0	−	40	2 hours
NS10	+	+	+	+	+	+	+	+	+	+	+	0	0	−	40	15 minutes
NS12	+	+	+	+	+	+	+	+	+	±	±	0	0	−	40	5 minutes
NS13	+	+	−	−	−	−	−	−	−	−	−	0	0	−	40	2 hours
NS14	−	−	−	−	−	−	−	−	−	−	−	0	0	−	40	2 hours

immune sera were prepared against fusiform bacilli. It is necessary, therefore, to use the serum within a few months after bleeding. While the titre drops rapidly, no prezone range has been observed.

Each strain of fusiform bacillus was tested against the four anti-sera. By means of these tests, three main types of fusiform bacilli have been found, together with two sub-groups. A fourth type has been added to the list, on the basis of its morphology only. This, the broad type already referred to, has a

characteristic morphology, differing considerably from that of the first three types. Due to its extremely meagre growth on surface cultures, no antigen could be prepared, hence this organism is included as a separate type only upon the basis of its morphology and character of growth on surface cultures.

No immune serum has been prepared against the wavy type of fusiform bacillus. It differs radically from the broad type,

TABLE 4

Agglutination reactions with Type I, Sub-type 2 serum

ANTIGEN	1:1	1:8	1:16	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	CONTROL	TEMPERATURE	TIME
															°C.	
LF1	+	+	+	+	±	–	–	–	–	–	–	0	0	–	40	2 hours
LW1	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
LW2	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
NW1	–	–	–	0	0	0	0	0	0	0	0	0	0	–	40	2 hours
LS1	+	+	±	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
LS3	+	±	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS4	–	–	–	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
LS5	±	±	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS6	±	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS1	+	+	+	+	+	+	+	+	+	±	±	–	–	–	40	2 hours
NS2	+	+	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	40	2 hours
NS10	±	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS12	+	±	–	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
NS13	+	+	+	+	+	+	+	+	+	+	+	0	0	–	40	5 minutes
NS14	–	0	0	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS5	+	+	+	+	+	+	+	+	+	+	+	0	0	+	56	5 minutes
NS1	+	+	+	+	+	+	+	+	–	–	–	0	0	–	45	2 hours

and since it fails to agglutinate with the sera of either types I or II, it is classed as a distinct type.

Results of the agglutination tests are given in table 1, which shows the type to which each of the cultures belongs. Sub-types are those which agglutinate in dilutions of 1:1 to 1:20, but which fail to agglutinate in dilutions greater than this. The other organisms listed all agglutinate with their type serum in dilutions of 1:2560 or higher, but not at all with sera of other types.

Type I organisms have been isolated most frequently from the cases studied, but these results are based on the study of too small a number of strains to enable one to draw any conclusions as to the relative frequency of occurrence of the various types. Type IV is very frequently seen in tartar, and it is also found in Vincent's angina, but it has been isolated in but two cases during this study. Further study will doubtless show a much greater

TABLE 5

Agglutination reactions with Type II serum

ANTIGEN	1:1	1:8	1:16	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	CONTROL	TEMPERATURE	TIME
															°C.	
LF1	–	–	–	–	–	–	–	–	–	–	–	–	0	–	40	2 hours
LW1	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
LW2	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
NW1	–	–	–	0	0	0	0	0	0	0	0	0	0	–	40	2 hours
LS1	–	–	–	–	–	–	–	0	0	0	0	0	0	–	40	2 hours
LS3	–	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS4	±	+	0	+	+	+	+	+	+	+	–	0	0	–	40	15 minutes
LS5	–	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
LS6	+	+	+	+	+	+	+	+	+	+	±	0	0	–	40	5 minutes
NS1	±	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS2	–	–	–	–	–	–	–	–	0	0	0	0	0	–	40	2 hours
NS8	–	–	–	–	–	–	–	–	–	–	0	0	0	–	40	2 hours
NS10	–	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS12	–	–	–	–	–	–	–	–	–	0	0	0	0	–	40	2 hours
NS13	–	–	–	–	–	–	–	–	–	–	–	0	0	–	40	2 hours
NS14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	40	2 hours
LS5	+	+	+	+	+	+	+	+	+	+	+	0	0	+	56	5 minutes
NS1	0	0	0	±	–	–	–	–	0	0	0	0	0	–	45	2 hours

percentage of the last three types than is here recorded, and may also show the existence of new types.

No relationship has been found between the type to which an organism belongs and the lesion from which it was secured. It is probable that no single type is constantly present in any one pathological condition.

DESCRIPTION OF TYPE CULTURES

Type I. Type organism LF1

In early generations bacilli of this type may be quite short, measuring 7 to 17μ in length, by 0.4 to 0.6μ in width, but in older generations they become filamentous and maintain this shape quite constantly.

The organisms are typically long, slender, and sharply pointed, often growing in long filaments which may attain a length of 225μ and a width of 0.5 to 0.65μ. Shorter forms are seen in all cultures. The average length is from 35 to 65μ. The shorter forms, which gradually disappear in successive generations, are from 4 to 19μ in length, and from 0.35 to 0.7μ in width.

Involution forms of various bizarre shapes are frequently found in cultures where the medium is unsuited to the growth of the organisms. Extremely filamentous organisms may be observed which are often over 300μ in length. These may be extremely tangled and twisted, so as to resemble a piece of tangled thread. As shown in figure 4, a slender organism may broaden out to a width of 3 to 5μ. Very large granules are sometimes found in these enlarged organisms. At other times, these forms may stain evenly and intensely. Grown on a medium which supports a vigorous growth of the organisms, these involution forms do not develop, and the culture dies out without their appearance.

No granules can be found in young, rapidly growing cultures when the organisms are stained with ordinary aniline dyes. Many granules may be seen, however, in cultures three to ten days old, in which degeneration forms have begun to appear. The protoplasm forms into granules, leaving a bare cell wall which shows as a hollow tube filled with the granules, which vary from one to fourteen. The first or second generation of a culture may contain many of these forms during the first twenty-four to forty-eight hours, but in later generations they do not appear until the culture is several days old. So constant is their appearance in old cultures as to suggest that their presence is a sign of decadence and approaching death and dissolution of the culture. Old stock cultures, stored several months without

transplanting, will also show many of these forms when freshly transplanted.

Single celled filaments outnumber all others, but at times filaments composed of from two to fourteen short, individual organisms of varying length, joined end to end, are seen. The juncture between the cells composing such filaments is often very indistinct, giving the appearance of a long, individual filament.

Observed under the dark field, the filaments appear to bend very stiffly. Single-celled filaments usually bend evenly, similar to a thin piece of steel, while multiple cell filaments may bend sharply, usually at the juncture of two cells.

The organisms stain readily with the strong aniline dyes, such as carbol-fuchsin or gentian violet. The former stain, diluted 1:10, is one of the best for the demonstration of granules. Stained with Atkin's modification of Gram's stain, and decolorized with acetone, the organisms are distinctly Gram-negative, no vestige of the original stain remaining.

Grown in liquid media, the bacilli clump together in huge masses of intertwined, filamentous organisms, even in fresh cultures, making such preparations unsuitable for agglutination purposes. Grown on solid media and suspended in saline, the organisms form an even, homogeneous suspension from which they settle out only after long periods of time. When so prepared, little difficulty due to spontaneous agglutination is experienced with Type I cultures.

Observed under the dark-field, no motility has been observed in any of the many cultures examined. No attempt has been made to stain flagella.

A very slight, characteristic odor, similar to that found in other types of fusiform bacilli, prevails in all cultures. This is by no means unpleasant. In impure cultures, however, a very foul, offensive odor quickly develops. As previously shown, this contamination is seldom detected by ordinary methods.

No sub-culture could be obtained from cultures exposed to the air in thin layers for twenty four hours, even after 34 generations of anaerobic surface cultures. No growth has been secured on repeated trial. Contrary to the results secured by

certain investigators, it is probable that the organism remains an obligate anaerobe, and that it is impossible to secure aerobic growths of pure cultures, even after prolonged cultivation.

In figure 1, a photograph of a freshly isolated, twenty-four-hour culture of this organism, granule formation is evident. As the organism becomes acclimated to artificial conditions of growth, granule formation in young cultures disappears. A nine-day culture of the same organism, in its twenty-seventh generation, is shown in figures 2 and 3. So-called shadow forms, in which can be seen numerous granules, are prominent in this photograph. The large clumps of filamentous organisms shown are commonly found in this type.

Fig. 1. B fusiformis, Type I. Second Generation, Twenty-four-hour Culture. × 1045 Diameters

Bacilli of Types I and II cannot be differentiated by means of their surface colonies, but these can be readily distinguished from those of contaminating organisms. Colonies on blood agar plates

FIG. 2. B. FUSIFORMIS, TYPE I. NINE-DAY CULTURE, TWENTY-NINTH GENERATION, SHOWING TYPICAL FILAMENTOUS CLUMPS, GRANULES AND "SHADOW" FORMS. 375 DIAMETERS

FIG. 3. B. FUSIFORMIS, TYPE I. SAME AS FIGURE 2, EXCEPT FOR THE MAGNIFICATION OF 1045 DIAMETERS

incubated in the phosphorus jar attain an average diameter of 0.8 mm. They are circular in outline, with a sharply defined, entire edge, rarely slightly indented. They are pulvinate in cross section. No fringe is ever present. Incubated anaerobically by Wright's method, they tend to spread out over the surface of the medium, forming thin, umbonate colonies 3 to 4 mm. in diameter, often with an indented margin, which are less characteristic than colonies grown in the phosphorus jar.

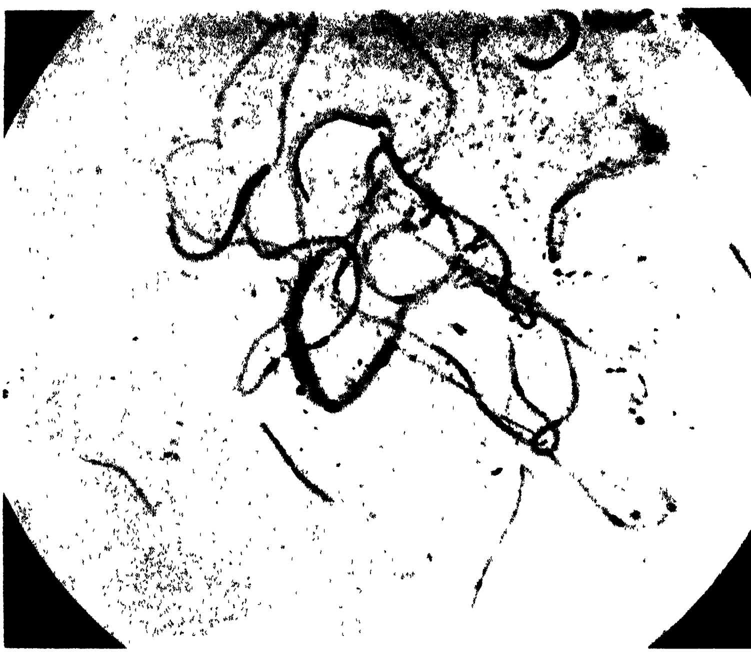

FIG. 4. B. FUSIFORMIS, TYPE I. INVOLUTION FORMS. × 1045 DIAMETERS

Extremely fine granular markings, which cannot be seen by strong light, appear on the surface of the colonies. Observed either by transmitted or reflected light, the interior of the colony appears to contain numerous white flecks within a water clear medium. This interior mottling is quite characteristic of Types I and II organisms, although it may be found in other types. Once recognized, the peculiar appearance produced by this mot-

tling aids greatly in isolating the organisms. One accustomed to their appearance can often pick the colonies on a plate by means of a naked eye examination alone, although colonies of certain streptococci may confuse one when they are examined in this way.

The organisms have a creamy white appearance in large masses, but no yellow color, as reported by a few investigators, has been observed. Thin layers of bacilli which have been exposed to the air for some time occasionally have a light violet tint.

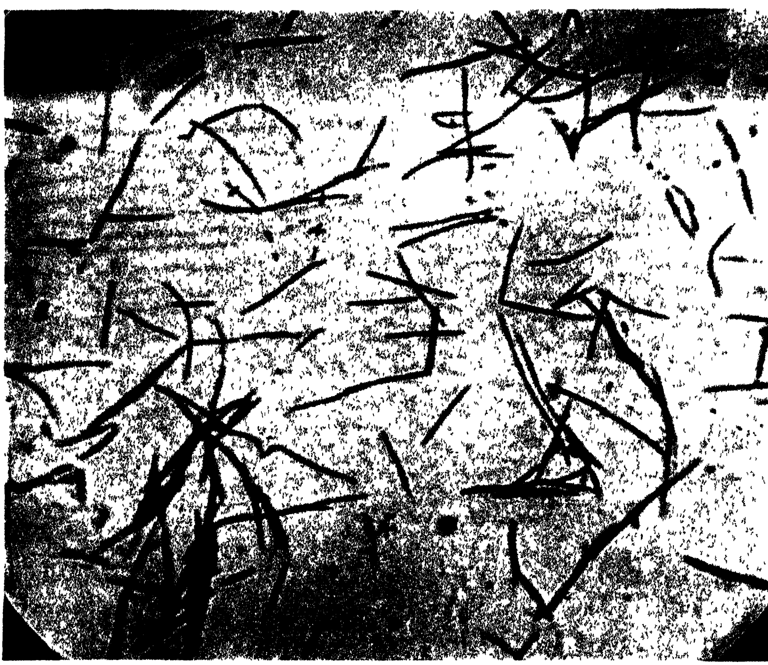

FIG. 5. B. FUSIFORMIS, TYPE I, SUB-TYPE 1. NORMAL FORM. THREE-DAY CULTURE, TWENTY-FIFTH GENERATION. ×1045 DIAMETERS

Type I. Sub-type 1. Type organism LS5

These organisms grow characteristically as long, straight, sharply pointed bacilli, with fewer curved forms than are found in LF1 cultures. Extreme differences in the length and breadth of the organisms, such as are found in the true filamentous type,

are not encountered in this sub-type. The characteristic form of the organism is shown in figure 5.

Individual bacilli vary in length from 9.4 to 19.3μ, and in width from 0.3 to 0.75μ, the average dimensions being 13.4μ by 0.5μ. Filamentous organisms 90μ long may be found on certain lots of media. Broad involution forms, such as are found in the culture previously described, are very rarely seen in this type. Nests of bacteria, which resemble masses of needle pointed crystals, frequently occur. While other types grow in this form, the longest individual organisms of any of these forms are found in sub-type 1 cultures. Chains of more than two bacilli are uncommon, although tandem forms are frequent. Out of hundreds of slides examined, but one chain of as many as five organisms was seen. This measured 10.5μ by 0.55μ, which is shorter than the average individual bacillus.

The bacilli stain rather weakly with gentian violet, but readily with 1:10 carbol-fuchsin. They are Gram-negative, and are readily decolorized.

In early generations, from two to eight granules form in each of the cells. As the organisms become more accustomed to artificial media granule formation in young cultures ceases to a large extent, occurring mainly in cultures from three to five days old. In such cultures, two, four or six granules form in each organism, with occasionally an odd number.

No motility has been observed in any of the cultures. A very slight odor, similar to that produced by other types of fusiform bacilli, is present in all cultures. No foul odor develops in pure cultures.

Surface colonies are indistinguishable from those of Type I cultures. The organisms are differentiated morphologically from those of the preceding type by reason of their length and tendency to grow in crystal-like masses. Serological tests should be used to identify the organisms positively, however, the morphology changing readily enough to make this an unsafe criterion of differentiation.

Homogeneous antigens are readily prepared from this type of organism, but these rapidly become granular. The organisms

agglutinate with Type I serum in dilutions below 1:20; against their homologous serum in dilutions of 1:10,240 or above.

Type I. Sub-type 2. Type organism NS13

The organisms belonging to sub-type 2 are the shortest of any found in Type I cultures. They are very sharply pointed, and occur characteristically in nests of organisms, similar to

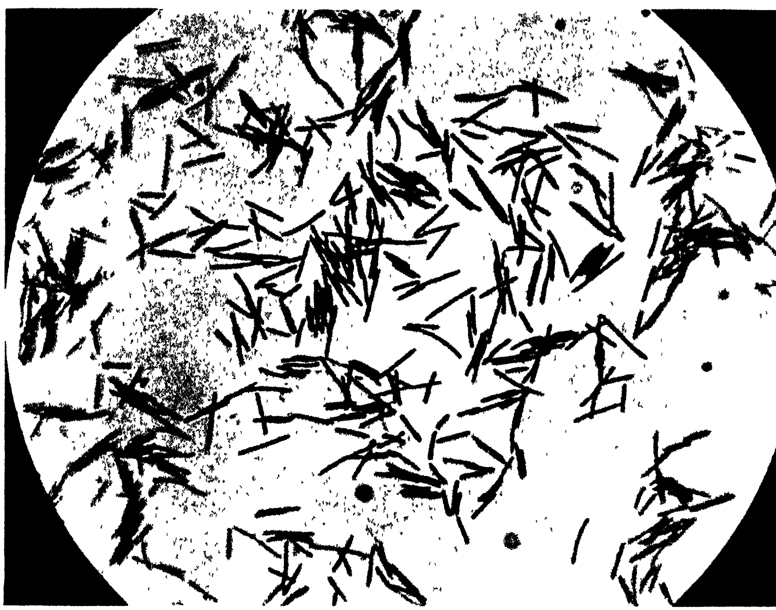

FIG. 6 B. FUSIFORMIS, TYPE I, SUB-TYPE 2. SECOND GENERATION, THREE-DAY CULTURE. × 1045 DIAMETERS

those of sub-type 1 cultures. Tandem forms, which may be slightly curved, are frequently seen. V type tandem forms are common. Single organisms are usually very straight.

In early generations short bacilli 4 to 12μ in length, by 0.5 to 0.7μ in width, are common. The average dimensions of these organisms are 7μ by 0.6μ. Later, both long and short forms are found, but the organisms never attain the lengths common to those of the filamentous type, varying from 2.8 to 19.5μ in length,

by 0.5 to 0.65μ in width. Occasionally short filaments as long as 45μ are found. The average size of the organisms found in older cultures is the same as in young cultures.

Individual organisms may contain from two to six granules. Shadow forms are seldom seen in this type, but when they do occur, usually contain from four to six granules. As with other

FIG. 7. B. FUSIFORMIS, TYPE I, SUB-TYPE 2. SECOND GENERATION, THREE-DAY CULTURE. EFFECT OF A SMALL AMOUNT OF ARSENIC IN MEDIUM. × 375 DIAMETERS

types of fusiform bacilli grown on surface cultures, very few granules form in young, rapidly growing cultures, although they may be present in great abundance in older cultures.

The organisms stain readily with strong aniline dyes, and are Gram-negative. They are non-motile, and give off a faint odor similar to that of other types of fusiform bacilli.

The characteristic morphology of this type of organism is shown in figure 6, in which the crystal like arrangement of the

organisms is apparent. The organisms shown in figure 7, which were grown on a medium containing a trace of arsenic, are of the same age and generation as those shown in figure 6, which were grown on an arsenic free blood medium. The effect of even a trace of arsenic on the development of fusiform bacilli is thus strikingly shown, illustrating the necessity of using an arsenic free medium.

FIG. 8. SAME AS FIGURE 7, EXCEPT FOR MAGNIFICATION OF 1045 DIAMETERS

Type II. Type organism NS14

These bacilli occur either as single organisms or in tandem formation. The ends are sharply pointed, although the juncture of two organisms growing in tandem form is blunt. In young, rapidly growing cultures the individual organisms vary in length from 2.3 to 5.1μ, and in width from 0.45 to 0.7μ. Tandem forms are seldom longer than the individual bacilli. The average dimensions of all forms is 3.9 by 0.58μ.

These organisms degenerate much less rapidly than those of other types so far studied, cultures six days old showing little or no signs of degeneration, excepting a slight increase in the length of the organisms. Cultures older than ten days contain numerous bacilli 10 to 12μ in length, which often grow in chains of two or more.

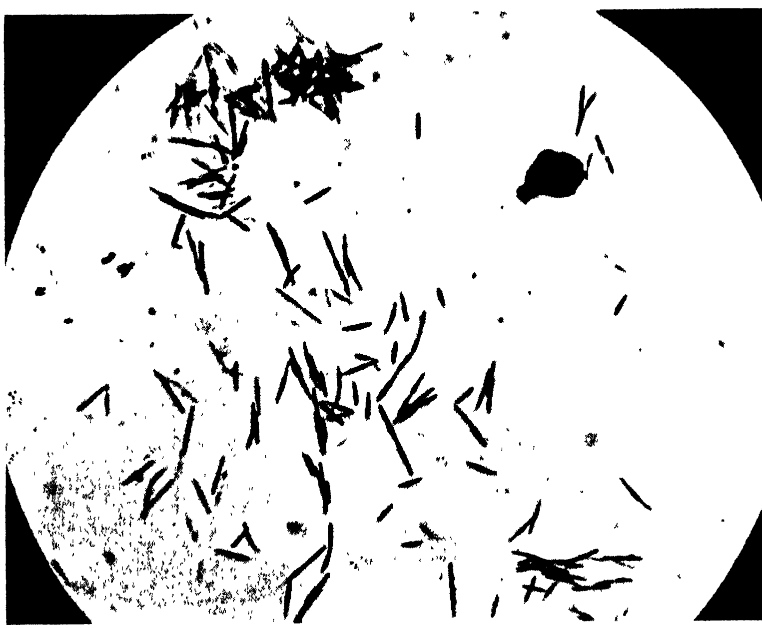

FIG. 9. B. FUSIFORMIS, TYPE II. THREE-DAY CULTURE, TWENTY-FIFTH GENERATION. TYPICAL APPEARANCE, SHOWING VERY SHORT FORMS. $\times$ 1045 DIAMETERS

The organisms are straight in young cultures, but in old cultures slightly curved forms are sometimes found, usually in tandem. In chains, the bend usually occurs at the juncture of two cells.

In old cultures short, granular shadow forms are present in large numbers, which, due to the presence of from two to four granules, bear a striking resemblance to diphtheria bacilli. Granules are rarely found in young cultures. Commonly but a single centrally located granule appears, which is usually of a

greater diameter than the cell. Tandem forms may contain either one or two granules.

The characteristic morphology of these organisms is shown in figure 9. Like the sub-types previously described, crystal like nests of bacilli are frequently found. Shorter individual bacilli are found in this type than in any of the other types studied.

FIG. 10. B. FUSIFORMIS, TYPE II. TWO-DAY CULTURE, FOURTEENTH GENERATION. ABNORMAL FORM, GROWN ON 2 PER CENT BLOOD AGAR. × 375 DIAMETERS

The effect of cultivating the organisms on an agar medium containing less blood than the organism is accustomed to is shown in figure 10. Less change of morphology is produced by this procedure than with Type I cultures. A pure culture of Type II fusiform bacillus was isolated from a tonsillar granule, from which the preparation shown in figure 11 was prepared. The effect of growing the organisms under artificial conditions is well illustrated by comparing this photograph with that of figure 9.

In their natural habitat, fusiform bacilli are less sharply pointed than when grown in pure culture, and are shorter and thicker.

The organisms stain rather poorly with gentian violet, but readily with 1:10 carbol-fuchsin. They are strictly Gram-negative. No motility has been observed in any of the cultures. A slight, characteristic odor prevails in all cultures.

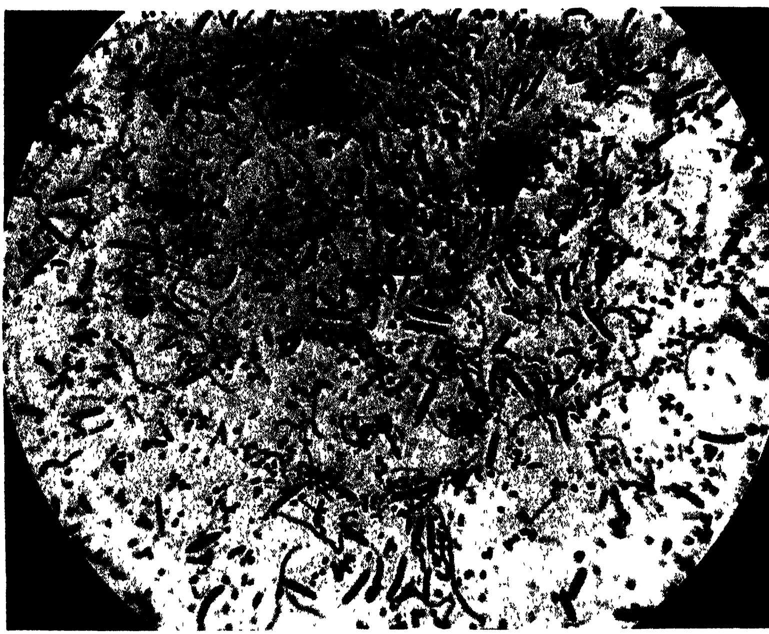

FIG. 11. FUSIFORM BACILLI AND SPIROCHETES FROM A DIRECT SMEAR OF A TONSILLAR GRANULE, FROM WHICH A TYPE II ORGANISM WAS ISOLATED. × 1045 DIAMETERS

The colony is similar to those previously described, with the same interior mottling. A small brown granular mass is sometimes seen in the center of the colony. On pushing aside the colony with a needle, the mass remains adhering to the media. A slight depression of the medium is produced beneath the colony, the same effect being noted with other types of fusiform bacilli. This can be seen only by washing the colony off the medium with saline.

The colony is slightly gelatinous and is hard to remove from the plate. No hemolysis of blood occurs in cultures kept under anaerobic conditions, but a slight hemolysis, due to hydrogen peroxide formation, is observed in cultures exposed to the air for some time.

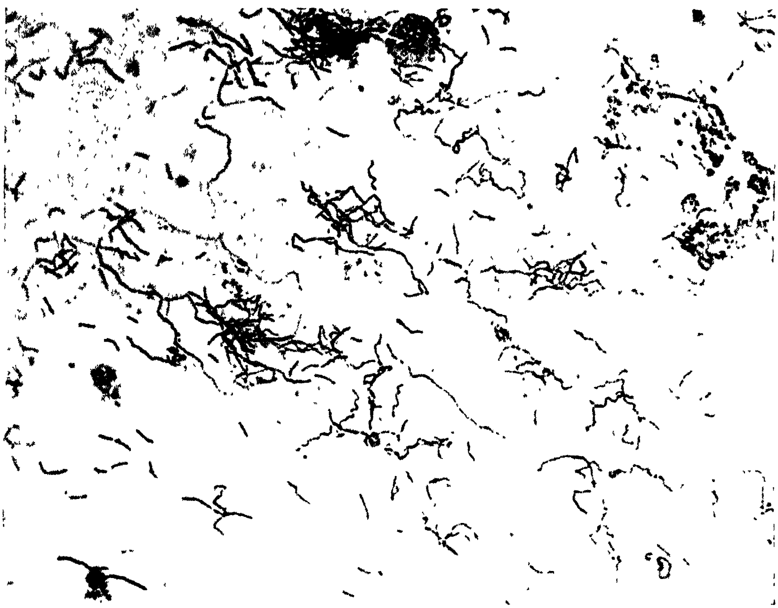

FIG. 12. B. FUSIFORMIS, TYPE III WAVY AND SPIRAL-LIKE FORMS, WHICH RESEMBLE SPIRILLA. SIXTH GENERATION, FORTY-EIGHT-HOUR CULTURE. × 375 DIAMETERS

Type III. Type organism LW1

Individual organisms of this type are less sharply pointed than are those of Types I and II. In twenty-four to seventy-two-hour cultures they appear characteristically in the form of long, wavy chains, as many as 35 individual organisms forming one chain. Chains 250μ in length are not uncommon. In chains, individual organisms longer than 37μ have not been seen. The majority are far shorter than this, varying from 4.2 to 7.1μ, with an average of 5.5μ. Extreme variations in width do not occur, the average being 0.5μ.

In cultures twenty-four to seventy-two hours old the organisms are extremely wavy, as illustrated in figures 12 and 15. These wavy forms often grow in huge clusters, as shown in figures 13 and 14. Some of the bacilli so closely resemble true spirilla as to be mistaken for them on cursory examination, or when they are examined under magnifications less than 1000 diameters. Under higher magnifications, these spiral forms can be seen to be

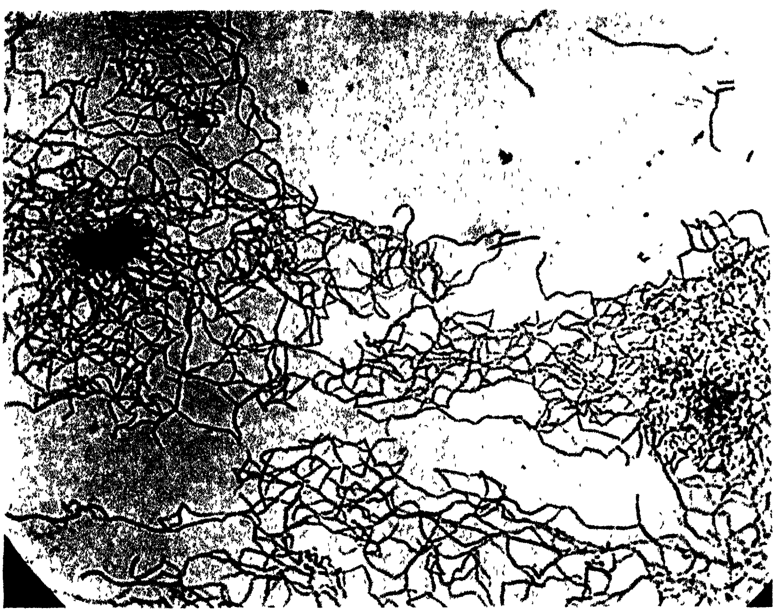

FIG. 13. B FUSIFORMIS, TYPE III SIXTH GENERATION. THREE-DAY CULTURE SHOWING CLUSTERS IN WHICH THESE ORGANISMS TYPICALLY GROW. × 375 DIAMETERS

composed of several individual fusiform bacilli, each of which forms a single curve of the spiral like element. Grown in liquid cultures, it is probable that this type of organism could not be differentiated from spirilla except by means of motility tests, so closely does it resemble this organism at certain stages of its growth.

In contrast to other types of fusiform bacilli, it is unusual to find perfectly straight organisms in young cultures. Some bacilli

may be but slightly curved; others, like those which form the spiral like elements, may be bent into the form of a half circle. So characteristic is this wavy or spiral-like form that a culture can be immediately identified by its appearance.

The spiral-like forms begin to disappear in cultures older than seventy-two hours, developing into straight or slightly curved, sharply pointed bacilli. These are slightly wider than young

FIG. 14 B. FUSIFORMIS, TYPE III. SIXTH GENERATION, TWO-DAY CULTURE, SHOWING TYPICAL SPIRAL-LIKE FORMS. × 1045 DIAMETERS

bacilli, having an average diameter of 0.7μ. Interspersed with these forms, which stain deeply, are numerous shadow forms.

No special stain has been needed to demonstrate these organisms, which is contrary to Tunnicliff's experience with her spiral-like forms. They stain readily with both gentian violet and 1:10 carbol-fuchsin at all periods of their growth. They are Gram-negative.

Two granules are usually found in the individual cells. In

old, degenerate forms, as many as six granules have been observed in a single organism. Two, however, are most commonly found.

No "external granules" have been observed in any of the many cultures examined, confirming in this respect similar observations made of all the other types of fusiform bacilli studied. In old cultures, a solidly staining organism lying end to end with a spiral-like shadow form is occasionally found, but there is no evi-

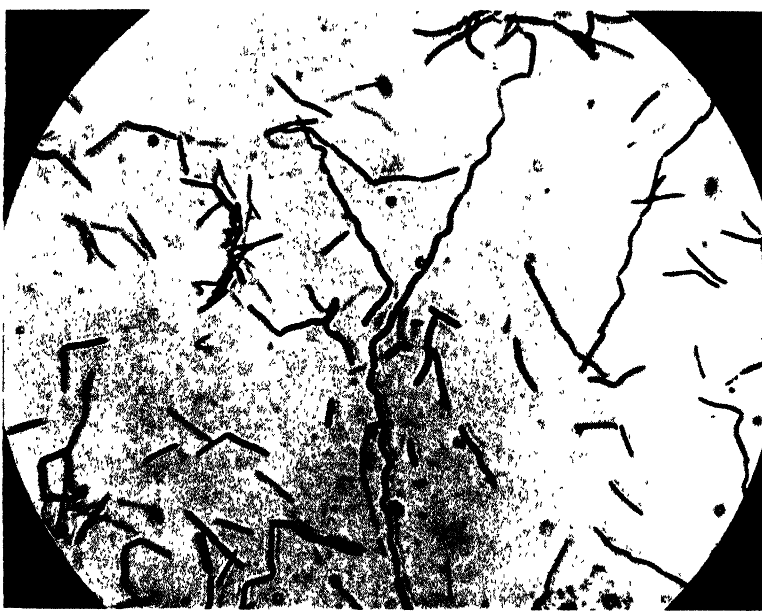

FIG. 15. B. FUSIFORMIS, TYPE III. TWO-DAY CULTURE. × 1045 DIAMETERS

dence that this spiral-like, weakly staining shadow form has emerged from the former. No evidence has been secured in the present study in support of the theory that spirilla may develop from granules which have dropped out of fusiform bacilli, or by the rearrangement of the protoplasm within the fusiform bacilli into spiral-like forms, which emerge from the cell as true spirilla through the breaking down of the cell wall. These theories are to be doubted. Efforts of several investigators to substantiate

them has led to a great deal of confusion in the study of fusiform bacilli. Careful surface culture study of this organism should readily clear up these disputed points.

No motility has been observed under the dark-field in any of the cultures, even in those showing a large number of spiral-like forms.

Colonies of Type III fusiform bacilli are slightly larger than those of the preceding types, the average diameter being 1 mm. They are perfectly circular in outline, with a sharply defined, entire edge. No fuzzy outgrowth is present. They are pulvinate in cross section, though slightly more rounded than colonies of Types I and II organisms. The surface is covered with a rather coarse granulation, giving to the colonies a pearly lustre, or an appearance which might be roughly described as resembling the surface of cast iron. Due to these markings, the interior mottling of the colony cannot be seen unless one first gently breaks it up with a needle. There are no central granular masses.

The colony is slightly viscid, and it is difficult to remove it from the surface of the medium. Heaped into irregular masses, the colonies soon flatten out.

In saline, an even, homogeneous suspension is formed, which settles out only after long periods of time. The organism does not agglutinate with any of the four immune sera prepared.

Type IV. Type organism LB1

Organisms of this type are much larger than those of preceding types, and have less sharply pointed ends, some organisms having blunt ends. They are frequently seen in direct smears from tartar, in which they occur as long, broad bacilli with pointed ends, sometimes staining evenly, sometimes unevenly. They are frequently found in tandem form, in which the two organisms are often of dissimilar size. Growing in short chains, the centrally located cells are often blunt ended, appearing sausage shaped. Again the ends may be square cut, the cells resembling chains of anthrax bacilli. In either case, the terminal organisms of the chain are pointed.

The bacilli vary in length from 3.8 to 17.4μ, with an average of

12μ. The shorter forms are found only in chains, never individually. Chains have been seen 54μ in length. The width is greater than that of the other three types, varying from 0.8 to 0.95μ, with an average of 0.9μ.

The organisms stain readily with aniline dyes, and are Gram-negative. Very minute, Gram-positive granules, unlike typical fusiform bacillus granules, are sometimes seen in a few of the

FIG. 16. B. FUSIFORMIS, TYPE IV. THREE-DAY CULTURE, THIRD GENERATION. × 1045 DIAMETERS

cells. These are found scattered throughout the entire cell, two or three often lying abreast. As many as fifteen have been counted in a single cell measuring 4.8μ in length. This "sprinkling" of small granules causes the organisms containing them to stand out sharply in a Gram-stained smear. Very few organisms contain them, however.

Degeneration sets in rapidly, beginning in forty-eight-hour cultures. The protoplasm shrinks away from the cell wall, forming into large, solidly staining granules regularly spaced. The cell wall between these granules is enlarged, and takes a very weak stain. This is shown in figure 16.

Due to the rapid degeneration of the surface cultures, and the extremely meagre growth obtained, no organisms belonging to this type have been kept alive for more than seven generations. A culture nine days old was the oldest from which a sub-culture could be obtained. Of the four types, this is by far the most difficult to isolate and culture.

The organisms are non-motile. Seen in liquid cultures, a mass of the organisms resemble a bunch of floating logs, so stick-like is their appearance.

An odor, similar to that formed by other types of fusiform bacilli, is given off by this type of organism. This is very faint, due, doubtless, to the small amount of growth present on a plate or slant.

Surface colonies are very thin and spreading, with an irregular margin. Seen with the naked eye, they resemble to some extent colonies of *B. tetani*. Well isolated colonies may attain a diameter of 3 to 4 mm. Closely spaced colonies show a marked diminution in size, few attaining a diameter greater than 1.5 to 2 mm.

The surface of the colony has distinct granular markings, while the edges are slightly curled. On surface culture, this type may be differentiated from those previously described not only by means of its morphology, but by means of its thin, spreading colony and meagre growth, in contrast to the sharply circumscribed colonies and heavy growth of the other types.

SUMMARY

From 18 pure cultures of fusiform bacilli, isolated by a new streak method, four different types have so far been identified by serological and morphological studies. Of these, Types III and IV can often be identified by morphological appearances alone, but the organisms of Types I and II, which vary greatly in their size and shape, can be safely differentiated from each

other only by serological tests. A classification of fusiform bacilli upon cultural and morphological grounds only should not be attempted.

Surface culture methods are adapted to the isolation and cultivation of all types of fusiform bacilli.

A wavy type of fusiform bacillus has been isolated, in which may be found spiral-like forms, so closely resembling true spirilla at certain stages of their growth as to lead to confusion. These spiral forms, which are non-motile and are present only for a short period of time, have no relationship to true spirilla.

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A TYPE OF UREA-SPLITTING BACTERIUM FOUND IN THE HUMAN INTESTINAL TRACT

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In the course of an investigation (1921) on the origin of ammonia dermatitis of the gluteal region of infants, it was shown that ammonia in the diaper is the result of a splitting of urinary urea by a bacterium which infests the gluteal region and diaper from the feces. This organism has been isolated from the stools of a number of infants and children and is probably one of the common saprophytes of the intestinal tract, not only in children but also in adults. Its occurrence in infants is of interest to the pediatrist on account of the not infrequent infestation of the gluteal region, with the production of ammonia in the wet napkin. In addition, its relatively widespread occurrence in the human intestine would indicate that the organism is one of the chief agents in the conversion of urea into ammonia in sewage. The marked urea-splitting action of this saprophyte, the fact that no record has been made of any previous study of the organism, and the observation that it is a common inhabitant of the human intestinal tract seem sufficient justification for recording the observations made in its study.

The conversion of urea into ammonia is a function possessed by a number of bacteria. The earlier observations of Pasteur, Miquel and others showed that various large spore-forming "urobacilli" could be isolated from air, soil, and sewage, and, later, many other bacteria were found able to form small amounts of ammonia from urea. No attempt will be made to discuss these ammonifiers except to mention that many Gram-negative bacilli are capable of producing small amounts of ammonia,

and that staphylococci also have this property. We wish to emphasize, however, that the organism here described differs from the above-mentioned strains of ammonifiers and especially from other organisms of the human gastro-intestinal flora, in being an exceptionally strong ammonia producer or urea fermenter. It is capable of growth and continued ammonia production in a medium so alkaline that other organisms are inhibited or destroyed. It seems probable that this particular bacterium has not been brought to the attention of other workers on the intestinal flora because suitable selective media were not used, and because their interest was not directed towards finding a strong ammonifier. Certain clinical observations on ammoniacal excoriation of the buttocks of infants prompted our search for a urea-splitting organism, and we were somewhat surprised to find that the form responsible was not one of the better known members of the intestinal flora. The only organism we have encountered having similar marked ability to form ammonia from urea is a yellow chromogenic sarcina, probably *S. lutea*, which has been found several times in air-contaminated ammoniacal urine after standing.

The organism here described was isolated in pure culture from the stools of more than 50 infants and older children, and was the only strong ammonia producer regularly found. *B. pyocyaneus* and *B. proteus*, both of which form moderate amounts of ammonia, were found a few times. The technique was simple and allowed an easy and rapid isolation. A small portion of the feces was inoculated into a tube of fresh, slightly acid urine, to which phenolsulphonephthalein (phenol red) had been added as an indicator, and which had been sterilized by passage through a diatomaceous filter. After incubation for twenty-four hours, if the reaction had become alkaline, several loopfuls were inoculated into a fresh tube of the same medium. When production of alkali had persisted through several tubes, some of the material was seeded on the surface of an agar plate. Colonies from this were transplanted into a urea medium consisting of 1 per cent urea and 0.2 per cent each of calcium chloride, monosodium phosphate, dipotassium phosphate and magnesium sulphate with

phenol red added as an indicator, and sterilized by filtration. Production of an alkaline reaction in this medium was always due to ammonia production.

The organism isolated has the following characteristics:

Morphologically, the cells from young cultures are non-motile rods with rounded ends and have a fairly uniform size. The majority have a diameter of 0.8 microns and a length which varies from 1.4 to 1.7 microns. A few shorter coccoid forms occur and occasionally an organism 2 to 3 microns long with a diameter of about one micron, but the large majority of elements are of almost uniform size. In older cultures, larger forms are somewhat more easily found and occasional irregularly pear-shaped organisms occur. There is also less uniformity in size. No characteristic grouping occurs and the morphology is similar on all culture media. Neither spores nor capsules occur. The organisms stain well and uniformily with the usual stains, retain the Gram stain, and are not acid fast.

On agar and gelatin the surface growth is filiform, fairly abundant, opaque, smooth, flat and glistening, with a butyrous consistency. No odor is produced and the medium remains unchanged. Gelatin is not liquefied. In stab cultures the growth is filiform and more abundant near the top. Although as a rule the cultures are not pigmented, several strains isolated have a faint yellow pigment. These chromogenic strains are otherwise indistinguishable from the non-chromogenic strains. Stab cultures give a filiform growth along the line of puncture which is more abundant near the surface.

The individual colonies on agar develop rapidly at 37°C. from 1 mm. diameter in twenty-four hours to 6 or 7 mm. in a week. They are round and flat, with a smooth surface and entire edge. The internal structure is amorphous.

In nutrient broth, cultures are without odor and show a moderate clouding, more marked in the upper layers, with an abundant flocculent sediment. Litmus milk is unchanged except for the production of a slight alkalinity. There is no diastatic action on starch jelly, blood serum is not liquefied, and none of the commonly used laboratory sugars are fermented.

Growth on all media is abundant at 37°C. although multiplication is somewhat more rapid at 30°C. The thermal death point is 55°C. in ten minutes. Cultures are rather resistant to drying and retain their vitality for several months in cultures. The most rapid development of cultures occurs on media with a reaction of pH 7.0 to pH 8.5 while greater degrees of acidity or alkalinity cause a marked inhibition of growth.

Considerable amounts of cultures were injected intravenously and subcutaneously into rabbits and guinea pigs without the production of any lesion.

The fermentation of urea is the function of this organism which has especially interested us and the following summarizes the results of observations on this property.

1. In unsealed cultures, urea is completely destroyed after a number of days, while in sealed flasks the destruction is incomplete. The alkalinity in such sealed flasks reaches a pH of 10 and at this point the bacterial growth is almost completely inhibited, although some viable organisms remain for a number of days.

2. No extracellular urease is present in the filtrates of young or old cultures.

3. Dried, powdered bacterial bodies contain a small amount of urease, usually considerably less than an equal weight of Jack Bean meal.

4. Salts of heavy metals such as copper and mercury, which have a marked inhibitory effect on urease activity even in very small amounts, do not inhibit ammonia formation in cultures unless added in amounts sufficient to prevent bacterial growth.

It is apparent that the formation of ammonia from urea is a function intimately associated with the vital activity of the organism, and does not depend on the formation of an extracellular ferment. In this respect it resembles other bacterial ferment activity.

According to the Committee of Classification of the Society of American Bacteriologists (1920), the organism belongs to Family 5, the Bacteriaceae, and in Genus 6, Bacterium. The name suggested is *Bacterium ammoniagenes*.

It may be remarked that the discovery of this organism in infants' stools, and of the mechanism of ammonia formation in the diaper with consequent dermatitis of the buttocks, suggested the cure of the condition by an impregnation of the diaper with an antiseptic which prevents further bacterial growth and ammonia formation. Thus an irritating skin eruption in infants, of common occurrence and sometimes rather distressing severity, has been completely controlled.

SUMMARY

The characteristics of a microörganism commonly found in the intestinal tract of infants and older children, and having exceptional power of splitting ammonia from urea, are described. The name *Bacterium ammoniagenes* is suggested for it.

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STUDIES ON AEROBIC BACTERIA COMMONLY CONCERNED IN THE DECOMPOSITION OF CELLULOSE

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I. INTRODUCTION

The decomposition of cellulose by microörganisms offers a field for investigation which has, for the past sixty years, been of interest from both purely scientific and practical standpoints. The natural decomposition of this resistant compound is not brought about by chemical processes alone, but is participated in by various biological agents and forces.

Cellulose is of almost universal distribution in the various tissues of plants, and forms a very large part of organic wastes. Unfortunately no way has yet been found for utilizing this ever-abundant product, except for a few limited purposes, as for example the manufacture of paper, nitrocellulose, etc. In nature the decomposition of cellulose goes on uncontrolled, and the products formed are immediately acted upon by microörganisms as sources of energy and food. In this way countless tons of decaying vegetable matter go to waste yearly. It might well be said that cellulose is almost an economic loss. If the biological process of decomposition could be carried on by pure culture methods and under scientific control, it should be possible to redeem the economically important cleavage products and convert them to every day use for man. Such mastery of nature is as yet only a dream, but from an analytical viewpoint it seems entirely possible.

Organisms varying widely in morphological, cultural and physiological properties have been associated by various workers with the natural decomposition of cellulose, namely certain

anaerobic and aerobic forms of bacteria, filamentous fungi, and some species of Actinomyces.

The painstaking investigations of van Iterson (1904), and of Kellerman, McBeth, Scales and Smith (1912–1916) show that aerobic bacilli play an important part in the decomposition. The present investigation deals with organisms of this group, some of which are transfers of the original Kellerman strains, while others are bacteria of similar type which have been isolated more recently, some of them by the authors.

In the present investigation attempts were made to simplify the isolation and cultural technique as a means of carrying out further studies on the activities of this interesting group of organisms.

II. GENERAL HISTORICAL REVIEW*

The first experimental evidence of cellulose decomposition by biological agents was advanced by E. Mitscherlich in 1850. He demonstrated that the cellulose parts of potato tissue were destroyed under certain definite laboratory conditions, and ascribed the change in structure to the action of certain vibrios found in the "substrate" of the potatoes.

Trecul (1865) published two papers on the microflora of macerated plant tissues. In these reports he discusses the activities of a group of organisms to which he applied the generic name *Amylobacter*. Van Tieghem (1877) later made an extensive study of the life history, cellulose-decomposing properties and physiological requirements of this group.

Popoff (1875) was the first worker to associate methane fermentation with the fermentation of cellulose. His experimental studies brought out the optimum temperature for the reaction and the effect of antiseptics, with some knowledge of the gaseous decomposition products.

Tappeiner (1881), Gayon (1883), and Deherain (1884), studied the various cleavage products of cellulose fermentation, observ-

* For a more complete historical review the reader is referred to the doctorate dissertation of the senior author, on deposit in the Yale University Library.

ing the formation of organic acids and numerous gases. The effects of oxygen supply, moisture, and composition of the medium on the nature of the by-products were also emphasized.

Hoppe-Seyler (1881), in an interesting series of experiments, came to the conclusion that cellulose decomposition goes through the following steps:

1. The hydration of the cellulose with the formation of a hexose.
2. The destruction of the monosaccharide with the formation of equal quantities of carbon-dioxide and methane.

Van Senus (1890) first suggested the phenomenon of symbiosis as entering into the decomposition process, and Herbert (1902) demonstrated that the speed of the fermentation was dependent to a large extent upon the reaction of the medium.

Schloessing (1889), Omelianski (1894), and Khanvine (1923) have contributed considerable data on anaerobic cellulose decomposition. The intensive studies of Omelianski cover a period of ten years and are especially interesting, since he was the first investigator to attempt work with pure cultures. He believed that cellulose could be decomposed by either of two fermentations, a hydrogen or a methane producing process, and that each process was influenced by a specific organism. In 1904 he published a method for separating the "hydrogen bacillus" from the "methane bacillus." His pure culture technique has been questioned, however, by later workers, since contaminating forms were isolated from his so-called pure cultures.

Meusel (1871), showed that certain organisms in the presence of cellulose are able to reduce nitrate to nitrite. Van Iterson (1904), in an extremely interesting study demonstrated that aerobic decomposition of cellulose is very vigorous, and that the oxygen requirements of the bacteria could be obtained from nitrates as well as from the atmosphere.

Pringsheim (1912), on the basis of experimental evidence, classified cellulose fermenting organisms as aerobes, denitrifiers, methane producers, hydrogen producers and thermophiles. His experiments with the cellulose-splitting enzyme are also striking.

The literature covering the years 1912–1916 inclusive contains a series of papers published by Kellerman and several co-workers (McBeth, Scales and Smith) as a result of experiments inaugurated by the Bureau of Plant Industry of the United States Department of Agriculture. The collective scope of these papers deals with the following phases of aerobic cellulose decomposition:

1. A discussion of the earlier work in the field by Omelianski and van Iterson, and the isolation of three strains of aerobic cellulose-fermenting organisms from the cultures of Omelianski.
2. The development of suitable mediums and technique for the isolation of aerobic cellulose-destroying organisms in pure cultures.
3. The collection of soil samples from various parts of the country, and subsequent isolation of over twenty strains of aerobic cellulose-decomposing bacteria and many filamentous fungi.
4. A description of the morphological, cultural and biochemical characteristics of these organisms.
5. A tentative classification of the group based on their cultural and biochemical properties.

Mutterlein (1913) developed a cellulose-agar medium which enabled him to isolate from soil cultures twenty strains of Actinomyces and one Bacterium which could destroy cellulose under aerobic conditions.

Kronlik (1913), using an ingenious technique, studied the activity of thermophilic cellulose-fermenting organisms under aerobic and anaerobic conditions. He concluded that the aerobic process is more rapid than the anaerobic, but that it is only through anaerobic conditions that complete destruction takes place. He also noted a difference in the gaseous end-products of the two types of fermentation.

Groenewege (1920), in an extensive study of the flora of aerobic cellulose fermentation, classified the organisms in three groups on the basis of nitrate reduction in the presence of cellulose. In a series of experiments he demonstrated the beneficial effects of nitrate-reducing organisms upon the action of the cellulose fermenters. Data are also given on the metabolic requirements of the cellulose-decomposing group.

The metabolic requirements of aerobic cellulose-decomposing bacteria were further studied by Löhnis and Lochhead (1922). They found that the organisms could utilize widely diversified forms of nitrogen, although there was evidence in favor of 0.1 per cent beef-extract as a nitrogenous source. Potassium nitrate seemed the best adapted source of inorganic nitrogen.

In the study of cellulose decomposition in soils by Waksman and Heukelekjan (1924), three methods for the quantitative measurement of fermentation were discussed, Charpentier's method being adopted as the most simple and the most accurate. This work also demonstrated the stimulative effect of sodium nitrate upon the decomposition of cellulose in soil.

III. SOURCES OF STRAINS EMPLOYED

Transplants of the following strains, originally isolated by Kellerman, McBeth, and Scales, were obtained from the Bureau of Plant Industry: *Bact. flavigenum*, *Bact. fimi*, *Bact. liquatum*, *Bact. udum*, *B. bibulus*, *B. biazoteus*, *B. gelidus*, *B. subalbus*.

Culture *B. x* is also an isolation of Kellerman and his associates, obtained directly from the Krall Laboratory. The label on the tube was not legible upon arrival of the culture, and to date the identity of the strain has not been determined.

Strains *8B*, *8G*, *5A*, *15*, *15N*, *16*, *Riv. 3* were isolated during the present investigation. Strains *8B* and *8G* were obtained from cow manure, *5A*, from compost, *15* and *15N* from decaying vegetable matter, and *16* and *Riv. 3* from soil.

IV. EXPERIMENTAL

According to the various publications cited the biological decomposition of cellulose is brought about by several morphologically distinct types of microörganisms; but it has been necessary to limit the present investigation to a study of aerobic cellulose-destroying bacteria. These organisms are able to attack cellulose under strictly aerobic conditions, or may carry out the oxidation with limited supplies of atmospheric oxygen if they are provided with nitrate or nitrite.

1. The collection and study of field specimens

In a preliminary experiment numerous samples of soil, decomposing vegetable matter, and sewage were collected for the purpose of studying the distribution of cellulose-destroying organisms in nature. It is obviously impossible to carry on an investigation of this kind unless sterile apparatus is used for the collection of each individual sample.

A very simple, inexpensive and convenient sampling outfit may be prepared from old cigar boxes and large test tubes in the following manner:

The boxes are split into one inch strips which are shaped into spatulas for collecting the soil. These are sterilized in the hot air oven along with large test tubes which serve as receptacles for the sample. Soil samples may be kept in good condition over a period of time by the frequent addition of sterile distilled water.

During the course of the experiment 39 samples were collected and examined in the laboratory for the presence of cellulose-decomposing bacteria. Approximately 1 gram of the soil was transferred to a tube containing the following enrichment medium:

Di-potassium phosphate	1 gram
Magnesium sulfate	1 gram
Sodium chloride	1 gram
Calcium carbonate	2 grams
Potassium nitrate	2 grams
Cellulose	strip of filter paper
Water	1000 cc.

It was assumed that the cellulose-destroying organisms were present in the inoculum when the strip of filter paper was completely cut at the surface of the liquid ten days after inoculation. In uninoculated control tubes the strips of paper were not broken after prolonged incubation, even after considerable handling and shaking.

Table 1 shows the different kinds of material collected and tested in the above medium, and the numbers of each in which cellulose-destroying organisms were demonstrated.

Although the limited number of samples collected does not allow any definite conclusion to be drawn concerning the distribution of cellulose-destroying organisms in nature, it may be said that this group seems to be closely associated with soils that are rich in decaying organic matter.

2. Special cultural mediums

Preliminary enrichment mediums for impure cultures. Although the destructive power of cellulose-fermenting bacteria was noted and described in 1850, it was not until 1912, or sixty-two years later, that pure cultures were isolated for study.

TABLE 1

Occurrence of cellulose-destroying organisms in nature

SOURCE	NUMBER OF SAMPLES	PRESENCE OF CELLULOSE-DESTROYING ORGANISMS INDICATED IN TEN DAYS
Garden soils	10	8
Forest soils	8	8
Decaying plant tissues	9	8
Swampy soils	3	3
Sewage	2	2
Humus heaps	2	2
Cow manure	3	3
Horse manure	2	2
Total	39	36

Various mediums and methods were tried, but all gave negative results. Their only value lay in showing the need of a preliminary enrichment medium for increasing the number of cellulose-destroying organisms before the final application of isolation technique.

Soils containing a large amount of organic matter are seeded with many types of organisms, and unless the chosen medium favors the growth of the delicate, slow-growing cellulose-fermenting bacteria, and at the same time retards the development of other forms, cellulose-destroyers are soon overgrown and isolation becomes impossible.

In 1894 Omelianski, employing Winogradski's "method of elective culture," selected for enrichment purposes a nutrient solution almost void of organic nitrogen, and incubated the cultures anaerobically, over a series of transfers. He found that this method gave almost a pure culture of cellulose-destroying organisms.

Van Iterson (1904), McBeth (1912), Löhnis and Lochhead (1913), and Groenewege (1920) have since suggested various modifications of Omelianski's solution, but the basic principle of the selective medium is essentially the same. No organic matter is introduced into the medium, except that contained in the inoculum. The nitrogen supply is limited to the inorganic salts of nitrogen. The cellulose-containing material (filter paper, cotton, plant tissues or commercial cellulose) comprises the sole source of carbon. Other inorganic salts, mainly those of sodium, magnesium and carbon, in the form of sulfates, phosphates, chlorides and carbonates, are added in varying concentrations to fulfil the metabolic requirements of the group.

After a preliminary study of various synthetic solutions, the Löhnis and Lochhead modification of McBeth's medium was chosen as the most satisfactory for enrichment purposes. The composition of this medium has already been stated on page 326, and it will hereafter be referred to as cellulose-nitrate broth.

This enrichment medium can be used with very satisfactory results under either of the following conditions:

1. Approximately 20 cc. of the nutrient solution and a strip of filter paper are placed in a large test tube. The filter paper is so placed that a portion of it projects about an inch above the surface of the liquid.

After sterilization the tubes are inoculated with 1 gram of soil and incubated at 34°C. until unmistakable signs of decomposition are noticed. Then a small piece of the disintegrating paper is aseptically transferred to a new tube of the same medium. Following a series of similar transfers, the cellulose-decomposing bacteria appear as the predominant type, and at this stage they are ready for transfer to the isolation medium.

2. Filter paper is shredded with dissecting needles and placed

in long-necked flasks. The flasks are then filled to the neck with cellulose-nitrate broth and sterilized. After sterilization, the cotton plugs are replaced by rubber stoppers. The flasks are inoculated with soil and the level of the liquid brought well up into the neck of the flask by the addition of sterile nutrient solution. Incubation is at 34°C. Fermentation appears slowly, but gradually increases in intensity. When the solution fails to give positive tests for either nitrate or nitrite, the old liquid is decanted off and new liquid is added aseptically. Small amounts of disintegrating cellulose are transferred to new flasks and the fermentation process repeated. After several such transfers, the cellulose-decomposing flora out-numbers the contaminating forms. Transfer to a solid medium for isolation requires only a small amount of inoculum.

Mediums for isolation. In a series of experiments the cellulose agar of Kellerman and McBeth was employed. Colonies of cellulose-dissolving bacteria could be easily detected on this medium by the appearance of a clear zone around the growing colonies. These areas have been referred to as the "enzymatic rings." Kellerman and others believe that they were formed by the action of a cellulose-decomposing enzyme produced by the bacteria within the colonies. Microscopic examination showed the areas to be free from cellulose fibers.

While using this medium considerable difficulty was experienced with molds which overcrowded the plates during the prolonged period of incubation. In an endeavor to overcome this difficulty by shortening the incubation period, casein-digest was substituted for the mineral constituents of Kellerman's medium. The results obtained with the modified medium were strikingly satisfactory. The incubation period was shortened from three to five days and the size of the colonies materially increased. Calcium carbonate was not added to the medium; yet the characteristic halo appeared around the colonies of cellulose-destroying organisms. This fact supports Kellerman's original claim that the clear zones around the colonies appear as a result of enzyme action, and not from the solution of calcium carbonate by acids produced during the fermentation.

Mediums for the cultivation of pure cultures. The problem of growing pure cultures of cellulose-fermenting bacteria is much more complex than the cultivation of the unpurified organisms. Cellulose-fermenting organisms will grow on ordinary laboratory mediums such as nutrient agar, gelatin, pepton meat-extract broth, and milk, but under these conditions some strains gradually lose their cellulose-fermenting powers or allow them to become dormant. Although Kellerman and others have devised means of rejuvenating this power from time to time, such a process solves the difficulty only temporarily. Several types of mediums that are useful in the study of these organisms have been devised, but they do not overcome the difficulties of continued cultivation.

A preliminary study made with the older methods revealed the following:

1. A diminution of the cellulose-fermenting power of the organisms on certain artificial mediums.
2. The production of delicate growths by these bacteria.
3. The necessity of long periods of incubation for obtaining maximum growth.
4. The irregularity of growth in cellulose-nitrate broth.

The results obtained in *cellulose-nitrate broth* were very irregular. In a few cases growth appeared in the initial transfer when large amounts of inoculum were used, but died out when a second subculture was attempted. This seemed to indicate that an inorganic medium was not favorable. The following experiment was carried out to determine the effect of the presence of organic matter upon growth.

A quantity of cellulose-nitrate broth was prepared in thoroughly washed glassware. The medium was divided into three equal portions; one of these was tubed and used as a control free from organic matter. Organic matter in the form of soluble casein-digest (Kulp and Rettger, 1924) was added to the other portions before tubing in final concentrations of 0.1 and 0.25 per cent respectively.

The three mediums were inoculated with suspensions of organisms which had been washed with saline solution to remove all

traces of organic matter. The inoculum was made up to a definite turbidity corresponding to 1.0 on the McFarland Nephelometer scale and added to the tubes in a 2 per cent concentration. Growth and change in hydrogen ion concentration of the various mediums is shown in table 2.

In a subsequent experiment no evidence of growth or decomposition could be obtained when the concentration of the inoculum was increased to 5 and 10 per cent.

TABLE 2

Growth of pure cultures with and without organic matter

ORGANISM	CELLULOSE-NITRATE (NO ORGANIC MATTER)		CELLULOSE-NITRATE (0 1 PER CENT ORGANIC MATTER)		CELLULOSE-NITRATE (0.25 PER CENT ORGANIC MATTER)	
	Paper cut after 8 days	pH after 8 days	Paper cut after 8 days	pH after 8 days	Paper cut after 8 days	pH after 8 days
Bact. udum	0	7 9	+	6 0	+	5.6
Bact. flavigenum	0	7 9	+	6 8	+	6 8
B. gelidus	0	8 1	0	7 4	x	7.0
B. subalbus	0	8 1	+	6 1	+	5 4
No. 15	0	8 1	+	7 4	+	7 0
Ps. perlurida	0	8 1	0	7 2	+	6 8
No. 8B	0	8 0	+	7 2	+	5 6
B. biazoteus	0	8 0	0	7 4	+	7.4
Riv. 3	0	7 9	+	6 0	+	6 0
B. x	0	8 1	+	6 4	+	5 6

0—Paper not cut after eight days incubation.
x—paper incompletely cut after eight days incubation.
+—paper completely cut after eight days incubation.

The results of these experiments seem to indicate the need of an organic medium for the continued growth of cellulose-decomposing organisms. All further attempts to cultivate them in inorganic medium were abandoned.

The tryptic digest of commercially pure casein, prepared by the method described by Kulp and Rettger (1924) has been shown in this laboratory to be superior to commercial pepton for the culture of certain organisms. Preliminary studies with the casein-digest showed that it was superior to commercial

pepton for the cultivation of cellulose-destroying bacteria. A *cellulose casein-digest medium* of the following composition was prepared:

Casein-digest	100 cc.
Tap water	900 cc.
Beef-extract	1 gram

The hydrogen ion concentration of the medium was adjusted to pH 7.4. A strip of filter paper was added to furnish the cellulose factor.

The cultural results obtained with this medium were very striking. When inoculated with either old or newly isolated strains, the organisms always grew. Definite changes in pH and turbidity could be demonstrated after twenty-four hours incubation. The strips of filter paper were attacked vigorously. The time required for a complete cutting of the paper varied from twenty-four to ninety-six hours.

The following experiment was carried out to determine whether the cellulose-fermenting power of the organisms would be altered by a series of successive transfers in this medium.

Six strains of cellulose-decomposing bacteria were employed over a series of nine transfers. The time necessary for complete cutting of the paper by each subculture, and the total number of days required to cut the nine strips of filter paper are given in table 3.

The length of the different trials varied from seventeen to twenty-seven days. This is probably explained by variation in the attacking powers of the different strains employed. At the end of the ninth transfer the cultures appeared to be as active in their growth and cellulose-fermenting properties as they were in the first subculture. Continual use of this medium during the past two years has furnished repeated verification of these results.

The advantages offered by the casein-digest medium for the cultivation of pure strains of these organisms may be summed up as follows:

1. It stimulates a rapid and luxuriant growth.

2. Cellulose is readily attacked and vigorously digested.
3. A very small amount of inoculum is required to stimulate growth.
4. The members of the group can utilize casein-digest without the presence of cellulose.
5. There has been no evidence that the different strains lose their cellulose-destroying activities.

Attempts to use this medium for enrichment purposes were without success, for it was non-selective and encouraged the growth of all organisms in a mixed culture.

TABLE 3

Cellulose-decomposition in cellulose casein-digest broth

ORGANISM	DAYS REQUIRED FOR CUTTING PAPER IN INDIVIDUAL SUBCULTURES									TOTAL DAYS REQUIRED
	1	2	3	4	5	6	7	8	9	
Bact. flavigenum	2	2	2	2	1	2	2	2	2	17
B. x.	2	2	2	2	1	2	2	2	2	17
No. 8B	2	2	2	2	2	2	2	2	2	18
Bact. udum	2	2	2	2	1	2	2	2	2	17
B. gelidus	3	4	3	3	2	4	3	3	2	27
No. 15	3	3	3	3	2	2	2	2	2	22

3. The isolation of pure cultures

The isolation technique of Kellerman, McBeth and Groenewege was tested with varying degrees of success. The methods of the above workers were then combined and modified in the preparation of a new technique that has worked satisfactorily during this investigation.

A small piece of fermenting cellulose (filter paper) was removed from the enrichment medium (cellulose nitrate broth) and carefully washed in sterile physiological saline solution. The decomposing material was then shaken vigorously in a tube containing sterile broken glass until it was completely disintegrated. At this point dilutions were made from the suspensions and plates poured, or the undiluted suspension was streaked over a series of plates containing cellulose casein-digest agar.

This modified method is believed to be an improvement over the others, for the following reasons:

1. The use of casein-digest as a nutrient solution, in the place of an inorganic medium, shortens the incubation period by stimulating the growth of the cellulose-fermenter.

2. The use of broken glass results in a more complete disintegration of the cellulose fibers, and likewise a more efficient separation of the bacterial cells attached to these fibers.

4. Morphological and cultural characteristics

Morphology. In the present investigation the strains under observation appeared as small, slender, non-spore-forming rods. Branching or chain formation was not observed. All strains stained readily with the ordinary basic dyes. When stained by Gram's Method they did not retain the stain after treatment with alcohol. All strains isolated during the investigation, with the exception of 8B and 8G, were motile at the time of isolation. Flagella stains were attempted, but did not give satisfactory results.

Cultural characters. These were studied in nutrient agar plates and slants, nutrient broth, litmus milk, potato slants, cellulose meat-extract broth, cellulose casein-digest broth, and maltose casein-digest agar plates. All cultures were incubated at 30° to 34°C.

Agar plates: The colonies formed in this medium varied from small (less than 1 mm. in diameter) to large (5 to 10 mm.) after fifteen days incubation. The surface colonies were round or almost round with well defined edges. The presence of a granular nucleus was not uncommon. Subsurface colonies were often granular, and lenticular in shape. A yellow chromogenesis was present in some strains and absent in others.

Nutrient broth: There was no surface film and only a slight amount of sediment formed. Slight to moderate turbidity appeared after from two to five days incubation. Odor was absent.

Agar slants: A moderate amount of filiform growth appeared after forty-eight to seventy-two hours. Chromogenesis appeared

in some strains. No discoloration of the surrounding medium was noted.

Litmus milk: Growth is slow. After from three to five days incubation, a slight reddening is noticeable in most of the cultures. Acid curds were not formed, but in one case a rennet curd appeared and was followed by peptonization. Litmus was not reduced. The cultures were incubated for fifteen days.

Potato slants: Eight of the cultures gave a sparse greyish-white growth along the line of inoculation; the scantiness of growth would indicate that the medium was not favorable for their cultivation.

Cellulose meat-extract broth: The type of growth in this medium was similar to that in nutrient broth. Cellulose was attacked after from three to five days.

Cellulose casein-digest broth: Cultivation of the various strains on this medium gave satisfactory results, both as to the amount of turbidity formed and the ability of the strains to attack cellulose. Twenty-four-hour cultures showed a distinct turbidity throughout the tube. In some cases a settling out of the organisms was noticed after from five to seven days.

Maltose casein-digest agar: This medium was found very useful for routine platings of the different strains. The amount of growth and the size of the colonies were greatly increased by the addition of the carbohydrate. The organisms were not allowed to remain in contact with this medium for long periods for fear of its altering their cellulose-fermenting properties.

5. Biochemical properties

Gelatinolytic action. The gelatinolytic action of the various strains was tested in ordinary gelatin stab cultures. These were incubated at 20°C. for thirty days. All of the strains tested grew in the medium, both on the surface and along the track of the inoculating needle. Liquifaction of the gelatin took place slowly and was generally infundibular in shape. After thirty days incubation all of the strains with the exception of no. 16 had produced liquifaction. Culture no. 16 gave a doubtful reaction after sixty days incubation.

Nitrate reduction. The organisms were cultivated in casein-

digest broth containing 0.2 per cent potassium nitrate. All gave a positive reaction for nitrite.

Indol production. None of the strains produced indol.

Fermentation studies. No reference could be found in the literature to changes in hydrogen ion concentration produced by aerobic cellulose-decomposing organisms. A series of experiments was carried out with the hope that fermentation reactions might show strain variations and thus furnish a basis of classification.

The basic medium employed was casein-digest broth. To this were added fermentable substances in the concentration of either 1.0 or 0.25 per cent. The majority of carbohydrates were sterilized by filtration through a sterile Berkefeld candle and then added aseptically to the basic medium. The detailed technique has been previously published by Kulp and Rettger (1924). The slightly soluble materials were prepared under aseptic conditions and subjected to a shortened sterilization period.

The determinations were carried out in triplicate. Uninoculated controls were frequently employed. The purity of the inoculum was tested by plating on maltose casein-digest agar.

The following test substances of the highest purity were used in the experiment:

Triose
- Erythrose

Pentoses
- Xylose
- Arabinose

Hexoses
- Glucose
- Levulose

Di-saccharides
- Maltose
- Lactose
- Sucrose

Tri-saccharides
- Melizitose
- Raffinose

Poly-saccharides
- Dextrin
- Soluble starch
- Inulin
- Cellulose

Glucosides
- Salicin
- Aesculin

Alcohols
- Gycerol
- Mannitol
- Dulcitol

The results obtained may be summed up as follows:

1. The lowest pH produced by any of the strains during the experiment was 4.6. A hydrogen ion concentration of from 4.6 to 4.8 seemed to be a maximum for all strains. The reactions varied both with the strains and the fermentable substances employed.
2. In several cases where no fermentation accompanied the growth of the organisms, alkali production could be noted by an increase in pH.
3. The alcohol, dulcitol, and the triose, erythrose, were the only two substances not attacked.
4. Inulin was attacked by only one strain, *Bacterium flavigenum.*
5. Mannitol, raffinose, and melizitose were attacked by only a few strains. Mannitol was fermented by *B. bibulus*, *B. x*, and *Ps. perlurida:* raffinose by *Bact. fimi*, *no. 15*, *B. biazoteus*, *Bact. liquatum* and *no. 15N;* and melizitose by *Bact. fimi*, *no. 15*, *Bact. liquatum*, and *no. 15N.*
6. The glucosides, aesculin and salicin, were attacked and fermented by all strains except *B. subalbus.*
7. The fermentation of glycerol was characterized by a small change in pH, except for a few strains. This substance did not appear readily fermentable.
8. Twelve of the seventeen strains used severed the filter paper (cellulose) within forty-eight hours incubation. Three more completed the cutting within the next twenty-four hours, and at the end of the fourth day all of the paper strips were completely cut.
9. All of the strains fermented the following:

Pentoses: Xylose and arabinose
Hexoses: Glucose and levulose
Di-saccharides: Maltose, lactose, and sucrose
Polysaccharides: Dextrin, soluble starch, and cellulose

The present investigation, although somewhat limited in its scope, due to the small number of available strains, points out the possibility of using carbohydrate fermentation as a means of differentiating various strains.

6. The Influence of Hydrogen Ion Concentration upon the Rate Of Cellulose Decomposition

Several workers have noted that cellulose-decomposing bacteria are very active when the reaction of the surrounding medium is neutral or slightly alkaline, but that the organisms are checked by the presence of small amounts of acid. A study was made of the changes in hydrogen ion concentration brought about by cellulose-destroying organisms in casein-digest broth. The results emphasized the apparent deterrent action of a slightly acid reaction in this medium. In a casein-digest broth having an initial pH of 4.9, growth and cellulose-decomposing powers were entirely inhibited. At pH 6.2 five out of the seventeen strains employed were able to grow and attack cellulose. All strains grew vigorously and attacked the cellulose when the initial pH was 7.4. In the last instance the pH was rapidly lowered during fermentation, and soon reached a point where activities were more or less hampered by the acidity of the medium.

Additional buffer material, in the form of secondary potassium phosphate, was added to the casein-digest medium in an effort to control the hydrogen ion concentration. The results indicate that 0.5 per cent phosphate in the medium exerts a buffer effect that holds the pH of the fermenting cultures above 6.5 for six days. In the control medium (casein-digest broth) and in a medium containing only 0.1 per cent phosphate the pH was considerably lowered during this incubation period.

7. Quantitative Determination of Cellulose Decomposition

In the present investigation decomposition was studied only in artificial cultural mediums. The basic medium employed was a casein-digest broth containing cellulose (strip of filter paper). Aside from the filter paper the medium contained no insoluble ingredients and passed readily through an ordinary filter.

The strips of paper used as a cellulose substrate were dried to constant weight before being added to the casein-digest broth. Paper and broth were sterilized together.

The amount of decomposed cellulose was determined by filter-

TABLE 4

Decomposition of cellulose in casein-digest broth containing a strip of filter paper

ORGANISMS	FILTER PAPER USED	FILTER PAPER DESTROYED DURING FIFTEEN DAYS INCUBATION	PER CENT DESTROYED
	mgm.	*mgm.*	
8B	254	30	12*
	255	29	11
	233	31	13
	227	32	14
	246	33	13
Bact. udum	255	35	14
	269	31	12
	259	29	11
	251	34	14
	260	34	13
B. gelidus	249	25	10
	257	30	12
	250	29	12
	252	28	11
	265	26	10
No. 16	244	22	9
	246	17	7
	267	28	10
	266	31	12
	262	24	9
Bact. flavigenum	266	22	8
	219	17	8
	246	28	11
	258	31	12
	265	24	9
B. x	277	8	3
	285	31	11
	266	38	14
	241	38	16
	246	34	14

* Figured to the nearest unit.

ing through a previously tared filter paper. The residue was thoroughly washed with distilled water to remove any trace of

soluble material. When secondary potassium phosphate had been added to the basic medium, it was necessary, first, to wash the residue with dilute hydrochloric acid (1:20).

Filter papers containing the residue, after washing, were dried to constant weight and the amount of decomposed cellulose readily calculated. Check weighings within three milligrams were required on all cultures. As a general rule four days drying in the oven at 110°C. was sufficient. Uninoculated control tubes were frequently employed and always registered within 5 mgm. of the calculated weight after being subjected to the above technique.

In the following experiment the cellulose-decomposing power of six strains was studied quantitatively in cellulose casein-digest broth. Five tubes of each strain were incubated simultaneously. The amount of paper destroyed by the various strains during the course of the experiment is shown in milligrams in table 4.

The effect of oxygen supply upon the decomposing powers of the organisms was studied in similar experiments. Reduced oxygen tension was produced by placing the cultures in glass-stoppered jars containing slant agar cultures of *B. cereus*. Increased oxygen supply was attained by gently aerating the cultures during the period of incubation. The results of the experiment indicated that neither of these measures increased the cellulose-decomposing properties of the organisms. Ten to 14 per cent of the cellulose was decomposed in both experiments.

The influence of secondary potassium phosphate upon the amount of cellulose decomposed received further attention here. Previous qualitative studies had shown that the presence of this salt tended to regulate the hydrogen ion concentration of the fermenting culture by its buffering action.

The basic medium employed was again casein-digest broth. The di-potassium phosphate was added in amounts giving concentrations of 0.5, 1.0, and 2.0 per cent respectively in three sets of flasks. The results indicate that the addition of the buffer materially affects the cellulose decomposition. Both one-half and 1 per cent concentrations of the salt favored destruc-

tion in almost every case. *B. gelidus* and *Bact. flavigenum* were the only exceptions, and this may be explained by the relative inactivity of these two strains.

The favorable effects of the phosphate appear to be lost when a 2 per cent concentration is used in the medium. There is approximately 100 per cent more cellulose destroyed in the medium containing 1 per cent phosphate than in one having 2 per cent of the salt. This retarding action is presumably due to growth-inhibiting action of the phosphate in the higher concentration.

V. GENERAL DISCUSSION

The results of the present investigation emphasize the necessity of a suitable culture medium for the artificial cultivation of pure strains of aerobic cellulose-fermenting organisms. In pure culture, as contrasted with the impure, the organisms are very delicate and require certain optimum conditions for growth and maintenance of physiological efficiency.

It was found impossible to obtain satisfactory results in the continued cultivation of the pure strains in nutrient solutions of inorganic salts. In most instances the bacteria refused to grow, and if they grew they rarely survived a second transfer. The addition of small amounts of soluble organic matter increased the growth possibilities.

The apparent beneficial effect of the presence of organic matter led to the development and use of a tryptic digest of chemically pure casein as a nutrient solution. Casein-digest has now been employed in this study for over three years, and it still supports luxuriant growth and permits vigorous cellulose fementation by the various strains employed.

Isolation of members of the group by the plating method was simplified by the adoption of a modified technique, and the substitution of casein-digest for the inorganc salt solution in McBeth's hydrocellulose agar. The period of incubation is materially shortened, and the size of the colonies increased. Calcium carbonate was not added to the medium; yet the "enzymatic ring" described by Kellerman and McBeth (1913) was clearly visible after incubation of the cultures.

The morphological and physiological properties of the cellulose-decomposing bacteria studied during this investigation were very similar, although the various strains came from widely different sources. The characteristics of the organisms suggest their classification in the genus *Cellulomonas* (Committee on Classification, 1920).

The organisms are very active in their attack on various fermentable substances. Xylose, arabinose, glucose, levulose, maltose, lactose, sucrose, dextrin, soluble starch and cellulose were fermented by all strains. Dulcitol and erythrose remained unattacked. Action on the other fermentable substances showed strain variation.

The results of quantitative studies emphasize the necessity of an optimum reaction for the fermentative process. The addition of buffer material to the basic medium tends to regulate the fermentation and also to increase the amount of cellulose destoyed.

VI. SUMMARY

1. Cellulose fermenting organisms appear well distributed in nature and are closely associated with decaying vegetable matter.
2. Unpurified cultures are most successfully cultivated in a selective inorganic nutrient solution. Pure cultures are best grown in an organic medium. Casein-digest proves very valuable for this purpose.
3. All but one of the strains are gelatinolytic; the one exception produces only slight liquefaction at best after prolonged incubation.
4. All strains reduce nitrate to nitrite.
5. Indol is not formed.
6. The fermentation reactions of the group suggest a possible means for strain differentiation.
7. The cellulose-fermenting powers of the strains may be demonstrated gravimetrically.
8. The addition of buffer in the form of secondary potassium phosphate (0.5 to 1.0 per cent) not only delays the development

of a harmful acid reaction, but also increases the actual amount of cellulose decomposition during fifteen days incubation.

9. The presence of the enzyme cellulase may be shown by the auxanographic method on cellulose casein-digest agar.

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THERMO-TOLERANT ORGANISMS AS A CAUSE OF SO-CALLED PIN-POINT COLONIES[1]

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INTRODUCTION

During the past decade the term "pin-point colony" has crept into bacteriological literature as descriptive of a certain type of small colony appearing on agar plates, especially in connection with milk analysis. Unfortunately the term is not well defined, but merely implies the existence of small colonies. Small colonies may be due to a wide variety of causes such as over crowding of plates, improper conditions of incubation, media requirements, etc., and not necessarily the result of the presence of a new and distinct type of bacteria in dairy products. If the term pin-point colony is to be used to designate an organism or group of organisms which characteristically produce very small colonies, it should be sufficiently well defined to prevent confusion with small colonies resulting from other causes. It is imperative, therefore, in order to discuss the subject, to come to some understanding as to just what constitutes the pin-point colony problem.

Bacteriologists for many years have observed very small colonies appearing in agar plates in the routine analysis of milk. If the colonies were large enough to count readily with the naked eye or with slight magnification, they were counted along with the other colonies and were given no further consideration. Within recent years, however, considerable complaint has been heard from laboratory workers, to the effect that pasteurized milk very

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frequently showed excessively high bacterial counts by the agar plate method. The observation seemed to be unanimous that in such plates from pasteurized milk the majority of the colonies were very small. Some of the colonies were so small that they could be seen only with the aid of a hand lens and would escape observation with the naked eye. The term "pin-point" seemed to be fairly descriptive of these colonies and has come into general use among laboratory workers who examine milk and other dairy products. The important and distinctive feature about these organisms is their ability to survive pasteurization, and not the characteristically small colony which they produce on agar plates. Were it not for the fact that these organisms could survive the process of pasteurization as commonly practiced, they would never have attracted more than a passing interest. The failure to recognize this thermo-resistant characteristic has led to a great deal of confusion in that some workers have described as "pin-points" any organism which possessed the characteristic of producing a small colony.

The pin-point colony problem may be defined as a condition observed in the bacteriological examination of milk, resulting from the presence of microscopic colonies of organisms capable of resisting pasteurization at 145°F. for thirty minutes. They are characterized by the production of very small colonies on thinly seeded plain agar plates, and are difficult to detect with the naked eye. It will be noted that in this definition the identity of any particular organism is not implied. As will be pointed out later in this paper, it is believed that there are several organisms capable of producing this type of colony under the specified conditions.

The significance and importance of the pin-point colony problem can be grasped at once when it is realized that the presence of such colonies in the agar plates threatens to minimize the value of one of the most widely used procedures in public health laboratories—the plating of milk. The interpretative value of bacterial counts of milk is based on the assumption that the conditions which are conducive to low bacterial numbers are the conditions under which milk should be handled. The large

number of organisms which may be present in a sample of milk do not, in themselves, necessarily render the milk unsafe; they are significant only in that the conditions which favored their introduction and multiplication are not in accordance with commonly accepted ideas of milk sanitation.

The pasteurization of milk usually reduces the bacterial numbers, as determined by the plate method, from 90 to 99 per cent, a fact which is frequently used as concrete evidence of the value of the process. However, if the flora of the milk is constituted of organisms which produce the so-called pin-point colonies, no such reduction will be observed. In fact, cases have been reported in which there was an appreciable increase in bacterial numbers during the process of pasteurization, indicating growth of these organisms at the temperature of pasteurization.

As far as pasteurized milk and dairy products are concerned, the presence of pin-point colonies in the plates may, therefore, lead to an incorrect interpretation of the sanitary quality of the product. Pasteurization is almost universally regarded as a desirable process for improving the safety of market milk, yet in the presence of these organisms, its efficiency could not be judged by the bacterial count. Yet public health officials are accustomed to judge the thoroughness of pasteurization by the per cent of bacterial destruction. Many cities have standards which stipulate the maximum bacterial content of milk prior and subsequent to pasteurization. The market milk plant operator who is unfortunate enough to have these heat resistant types of bacteria well established in his plant, soon finds himself unable to meet the demands of the standards relative to bacterial numbers. In spite of the fact that the milk has been thoroughly pasteurized at 145°F. for thirty minutes, and its sanitary quality has been improved through the destruction of any disease producing bacteria which may have been present, the total bacterial count may still be nearly as high as that of the raw milk. When the milk is judged on a basis of its bacterial count, the improvement in its sanitary quality through pasteurization may not be recognized, if these organisms constitute a major part of the flora.

REVIEW OF LITERATURE

Many instances are reported of the isolation of thermo-resistant and thermophilic bacteria from milk. In fact it is possible to demonstrate the presence of such organisms in almost every sample of mixed market milk. The omnipresence of these types is usually considered to be of little significance, since they are usually present in small numbers and seldom find conditions favorable for their rapid multiplication. For a thermophilic organism to be of significance in milk, it must be able to grow rapidly under the conditions under which milk is usually handled. If an organism is capable of growing during pasteurization its presence in the milk supply or the equipment becomes a serious problem.

The literature on thermophilic and thermo-resistant types of bacteria is excellently reviewed by Morrison and Tanner (1922), and for this reason a detailed review of the literature on the thermophilic bacteria is not presented here.

Perhaps the first report of the pin-point colony problem was made by Jacobsen (1918), in connection with a study made in two Oregon dairies. His study of the reasons for wide variations in the bacterial content of milk coming from these two plants revealed the fact that in some cases the pasteurized milk from one of the plants had a higher bacterial count than the raw milk. Pasteurization of some of this milk at 144°F. for forty minutes under laboratory conditions showed bacterial counts of 197,000 before, and 246,000 after heating. The trouble reported by Jacobsen coincided with the experiences of many workers in milk laboratories, and although no definite reports were made, observations of this type were common. At the Kansas Experiment Station, this phenomenon appeared in 1921 in connection with some consecutive pasteurization experiments with ice cream mix.

In an article by Ayers and Mudge (1920) appears the statement: "There are certain types of lactic acid bacteria which grow slowly on extract agar, and their colonies may be either just visible or not show at all after forty-eight hours incubation at 37°C."

At the meeting of the Society of American Bacteriologists in 1922, two papers dealing with this subject were presented. Harding (1923) reported results of bacterial counts on samples taken from a continuous pasteurizer at frequent intervals during the day. A very marked rise was noted during the day's run. Yates (1923) described the high counts obtained from the pasteurized milk supply of Kansas City, Missouri as due largely to the presence of "pin-point colonies." He believed these were associated, and coincident with, the advent of the hydrogen-ion method of media standardization. His data also led him to believe that the outbreaks might have been associated with the use of chlorine solutions in sterilization of equipment. Ayers and Johnson (1923) encountered pin-point colonies in their study of the transportation of heated milk.

A study of the heat resistant organisms surviving pasteurization led Robertson (1923) to conclude that they had little to do with the keeping quality of the milk, since most of his cultures failed to produce sufficient acid to curdle the milk.

The general agitation among laboratory workers who had observed this phenomenon was brought to a focus at the meeting of the Society of American Bacteriologists in New Haven in 1923. Taylor (1924) submitted data showing the gradual increase in the count of pasteurized milk delivered from a continuous flow pasteurizer. Hungerford and Harding (1924) presented figures which also indicated the tendency of milk from a continuous flow pasteurizer to contain large numbers towards the end of the day. Repetition of this experiment for twenty days convinced these investigators that the most probable explanation was that growth of bacteria took place in the pasteurizer.

As a result of incubating 235 samples of milk at 145°F. for 3.5 to 24 hours, Adams and Harding (1924) demonstrated thermophiles in 28.2 per cent of raw, common milk; 43.7 per cent of class A milk, and 40.4 per cent of certified milk.

Harding and Ward (1924) incubated at high temperatures composite samples of pasteurized milk taken directly from pasteurizing tanks. They found that in each of 12 cases there was an increase in the number of bacteria over the count made immediately after pasteurization.

Tanner (1924a) concluded that thermophilic bacteria were not abundant in milk, but that they could be readily demonstrated after an enrichment period of twenty-four hours incubation at 55°C.

Tanner (1924b), in another article, reported the isolation from milk of the thermophiles which were capable of growing at pasteurizing temperatures. He called attention to the likelihood of the presence of these organisms causing misinterpretation of total bacterial counts on pasteurized milk.

Cooledge (1924) believed the appearance of pin-point colonies was associated with the reaction of the media. The same sample of milk plated on two media with reactions of pH 6.6 and 7.3, respectively, resulted in counts of 15,400 per cubic centimeter on the former, and 317,000 per cubic centimeter on the latter medium; the difference being due to the appearance of pin-point colonies. Cooledge further suggested that the appearance of these colonies in low dilutions was due to the ability of the large number of bacteria present to change the reaction of the media sufficiently to permit their development.

Yates and Glover (1924) in a comparison of pre-war media with Bacto-agar found that the latter gave higher counts with pasteurized milk. They believed that the pin-point colony phenomenon was not affected so much by the reaction of the medium as by the ingredients used in its preparation.

Van Horn (1924) found that pasteurized milk showed about three times as many colonies on Difco beef extract agar as when Liebig's beef extract was used. The raw milk, on the other hand, gave counts only slightly higher when the Difco extract was used.

Ayers and Johnson (1924) suggested the name *Lactobacillus thermophilus* for a Gram-positive, aerobic, non-spore-bearing rod which they found to be the cause of an outbreak of pin-point colonies in the analysis of milk from a commercial pasteurizing plant. The organism which they isolated in 37 out of 39 cultures had an optimum temperature between 50° and 62.8°C. and grew rapidly at pasteurizing temperature.

H. G. Harding (1925), in a thesis presented at the University of Illinois gave an excellent review of the literature bearing on the

subject of thermophilic bacteria in milk, as well as on the pin-point colony problem. He found the thermophilic flora of milk taken from the udder to be very small. Harding also found no relationship between the number of bacteria appearing on duplicate plates incubated at 37°, and at 50°C.

Swenarton (1925) expressed the opinion that pin-point colonies might be due to a variety of causes. Observations, based on data obtained in the bacteriological analysis of the Baltimore milk supply, showed that this phenomenon was most prevalent in the spring of the year. Of 52 cultures which this investigator isolated from typical plates, 96 per cent were streptococcus types. He suggested a possible relationship of these organisms to mastitis.

Johnson and Exworthy (1925) made isolations from pin-point colonies and found them to be thermo-resistant streptococci. The cultures isolated survived 62.5°C., and grew between 25° and 50°C. The authors concluded that the thermophilic streptococcus which they isolated was one of the causes of pin-point colonies.

Harding and Ward (1926) did not believe that the appearance of thermophilic bacteria in a milk supply could be attributed to any recognized factor. Their extensive observations, covering many city milk supplies, led them to conclude that the season of the year was not a factor in the pin-point colony problem.

Tanner and Harding (1926) were able to demonstrate thermophilic bacteria in every sample of milk examined. All the cultures which they isolated were motile, Gram-positive, spore-bearing rods which grew well at pasteurizing temperature (62.5°C.).

Fay (1926) observed pin-point colonies in the low dilution plates made in the bacteriological analysis of ice cream. These were shown to be thermo-resistant, but not thermophilic. Morphologically they were Gram-positive, short oval rods, resembling *S. lactis* in arrangement.

Thermo-tolerant organisms differ from true thermophilic types in that they are able to tolerate high temperatures but do not multiply at high temperatures. The so-called thermo-tolerant organisms are capable of withstanding for several hours a temperature which would kill most vegetative cells in a few minutes.

PRELIMINARY OBSERVATIONS

In previous experimental work at this station it has been frequently observed that successive pasteurization of ice cream mix and other dairy products occasionally failed to reduce the number of viable organisms. Approximately the same bacterial count would be obtained after the first pasteurization. The colonies appearing on the plates were very small, sometimes even escaping the observer's notice until examined with a hand lens. Another puzzling observation was the fact that such colonies appeared only on the low dilutions (1:10 and 1:100), none developing on the 1:1000 and higher dilutions. Swenarton (1925), in his study of the pin-point colony problem in the Baltimore milk supply, likewise observed that the highest counts were observed in the low dilutions.

The following is a specific instance representative of many subsequent observations. In examining the agar plates from an ice cream scoring contest, one sample had the rather low count of 2900 bacteria per gram. The 1:10 dilution plates averaged 285 colonies of regular size, the 1:100 dilution plates 30 colonies, and the 1:1000 dilution plates four colonies, the numbers indicating reasonably accurate dilutions. The plates were reëxamined more carefully with a hand lens to verify the low count, and on careful scrutiny it was observed that there were myriads of very small, clear colonies in the 1:10 dilution plates, which had entirely escaped previous notice. An attempt to estimate the number of small colonies indicated that there were approximately 20,000 of them in each plate. Examination of the 1:100 dilution plates showed that there were about 3000 of the small colonies present, which checked fairly well with the 1:10 dilution, in view of the fact that it was not feasible to make an actual count. From this it would be reasonable to expect approximately 200 to 300 of the small colonies to appear on the 1:1000 dilution, and 20 to 30 on the 1:10,000 dilution plates. On very careful examination of these plates, however, not a single pin-point colony could be found. Obviously, there must have been 200 to 300 organisms introduced into the 1:1000 plate, since the regular sized colonies

were present in expected numbers. For some reason, however, conditions were not favorable for the development of the organisms responsible for the pin-point colonies in the higher dilutions. This chance observation changed the count on the ice cream from 2900 to 300,000 per gram and, incidentally, made a difference of 20 points in the score of the product in the contest. This ice cream was made in one of the largest and best equipped ice cream plants in Kansas. Sanitary conditions were excellent and every effort was made to produce ice cream with a low bacterial content. It was the practice in this plant to pasteurize the mix at 165°F. for twenty minutes. In view of the special care given this contest sample it is reasonable to assume that the organisms survived pasteurization and did not gain access during subsequent handling. A sample of the same ice cream was submitted to another laboratory before the contest, and a bacterial count of 2400 per cubic centimeter was reported. Many such instances have been observed in connection with the work at this station and, no doubt, have been noted by many workers elsewhere.

Apparently there is something in the low dilution plates which supplies the requirements for the growth of pin-point colonies that is not present in high dilutions. Re-plating such samples on plain agar, to which had been added carbohydrate in the quantity which would be introduced with a 1:100 dilution of ice cream mix, made the conditions suitable for the development of the expected number of pin-point colonies in the high dilutions. When the amount of carbohydrate added was materially less than the quantity introduced with a 1:100 dilution of ice cream mix, the requirements for growth were not satisfied. This explains the development of 3000 small colonies on the 1:100 dilution in the example just cited, and the failure of the 200 to 300 expected colonies on the 1:100 dilution to appear. The addition of comparable quantities of gelatin, casein or butter fat did not induce growth of pin-point colonies in the high dilutions.

Attempts to grow these organisms at the temperature of pasteurization (63°) repeatedly failed with all the cultures isolated. They would survive the temperature of pasteurization,

however, for several hours. Milk inoculated with them could be repeatedly pasteurized without material reduction in bacterial numbers. In that they did not grow at pasteurizing temperature they do not correspond to the description which Ayers and Johnson (1924) give of pin-point colonies, yet their ability to survive pasteurization, and other typical characteristics which they possess render them a part of the pin-point colony problem.

Advantage was taken in isolating these organisms of their ability to survive several pasteurizations. Samples of milk and other dairy products were pasteurized in flasks by means of a water bath at 63°C. for thirty minutes, and cooled to room temperature. This was repeated several times until, in some cases, the samples had been pasteurized nine times. In all cases they were pasteurized at least three times. Agar plates were made before and after each pasteurization. From these the presence of heat resistant pin-point types could be detected, and if found they could be readily isolated.

Before the first pasteurization, a portion of the milk was set aside in an incubator at 63°C. for approximately eight hours, after which it was plated on carbohydrate media. By this method it was hoped to detect not only organisms which were sufficiently thermo-tolerant to survive several pasteurizations but also organisms which were sufficiently thermophilic to grow at pasteurizing temperature. The first attempts resulted in the isolation of several "pin-point" cultures from the pasteurized milk.

Duplicate plates were made from the incubated milk and one of these was incubated at 63°C. and the other at 37°C. The results on both plates showed the presence of thermophilic bacteria producing large colonies which developed on both plates; but none of the typical "pin-point" types could be found. Enrichment periods of twenty-four and forty-eight hours at 63°C. were then tried, but with the same results. After failure to isolate "pin-point" types from about 20 samples, the temperature of enrichment was reduced to 56°C., and later to 47°C., with much better results. After isolating from several samples, both by the consecutive pasteurization method, and also by enrichment

at 47°C., it was noted that by the latter method all the samples containing the desired types could be detected. Since it involved much less time and material, the enrichment method was adopted in all succeeding work. Small colonies which conformed to the general idea of "pin-points" were selected and inoculated into litmus milk or brom-cresol-purple milk.

EXPERIMENTAL WORK

Resistance of isolated cultures to pasteurization

The isolated cultures were inoculated into flasks of perfectly fresh whole milk which had been previously heated for thirty minutes at 100°C. From 3 to 5 cc. of a twenty-four-hour milk culture of "pin-points" were added to 100 cc. of heated fresh milk. After thorough agitation the inoculated milk was sampled for plating, then pasteurized in a carefully controlled water bath at 63°C. for thirty minutes. In order to approximate vat pasteurization more closely, the heating and cooling of the samples were conducted in such a manner that it required twenty minutes to reach 63°C. and twenty minutes to cool to room temperature. The samples were agitated during the heating period. As soon as one pasteurization was completed and the milk cooled, the flasks were sampled for plating, and the pasteurization immediately started again. One of the cultures was pasteurized in this manner eight times, five were pasteurized seven times, and the remainder were pasteurized three times. Referring to table 1, which shows the heat resistance of these cultures to pasteurization, it will be noted that an average of 99.84 per cent of the organisms survived the first pasteurization, 72.01 per cent of the second, and 46.33 per cent the third. When pasteurization was continued with six of the cultures, 37.3 per cent of the organisms survived the fourth pasteurization; 26.6 per cent the fifth; 15.2 per cent the sixth, and 4.5 per cent the seventh pasteurization. The sample which was pasteurized eight times was sterile.

It will be noted that in some cases the results show an apparent increase in bacterial numbers after pasteurization. With one possible exception (no. 17), it is believed that these are within the

TABLE 1

Effect of successive pasteurizations on pure cultures from pin-point colonies

EXPERIMENT NUMBER	PER CENT SURVIVING AFTER							
	First pasteurization	Second pasteurization	Third pasteurization	Fourth pasteurization	Fifth pasteurization	Sixth pasteurization	Seventh pasteurization	Eighth pasteurization
1	94.8	30.1						
2	46.6	21.3	20.0					
3	114.2	115.4	83.3					
4	54.5	31.8	0.6					
5	111.1	100.0	10.0					
6	104.6	100.0	115.3					
7	105.2	105.2	13.1					
8	88.8	96.2	103.7	74.0	70.3	70.3	11.1	
9	69.6	9.0	0.1					
10	100.0	63.6	36.3					
11	111.7	64.7	58.8					
12	81.2	68.7	68.8	31.2	9.3	1.8	3.1	
13	100.0	100.0	40.0					
14	90.9	63.6	54.6	13.6	3.6	0.1	0.0	
15	127.7	66.6	77.7	77.7	66.7	22.2	11.1	
16	84.6	46.1	5.3					
17	105.8	82.3	223.5					
18	60 0	50.0	40.0	13.0	4.0	0.8	1.6	
19	100.0	85.7	0.1					
20	100.0	85.7	35.7					
21	83.3	75 0	75.0					
22	85.7	71.4	11.4					
23	100.0	100.0	1.3					
24	84.2	68.4	9.4					
25	100.0	60.0	30.0					
26	68.0	72.3	78.7					
27	100.0	100.0	41.6					
28	106.0	69.6	9.0					
29	112.5	75.0	12.5					
30	133.3	53.3	60.0					
31	100.0	83.3	3.0					
32	100.0	100.0	100.0					
33	100.0	71.4	71.4					
34	88 2	29.4	22.0	14.7	4.4	0.7	0	0
Average..	99.84	72.01	46.33	37.36	26.68	15.20	4.52	0

limits of experimental error. Comparatively wide variations were frequently unavoidable due to the fact that the colonies were small and difficult to see. In culture no. 17 an increase of 223.5

per cent is reported after the third pasteurization. This at first was regarded as evidence of the ability of the culture to grow during pasteurization. However, later attempts to culture the organism at 63°C. repeatedly failed.

After the first pasteurization, there were 20 out of 34 cultures in which the number of organisms was not reduced at all, and eight of these were not affected by the second pasteurization. After the third pasteurization, however, many of the cultures began to decrease in number. Only eight of the cultures had more than 75 per cent of the original number after the third heating, and 19 of them had lost more than half their number. It is interesting to

TABLE 2

Frequency of isolation of pin-point colonies from dairy products

PRODUCT	NUMBER OF SAMPLES	ISOLATION OF PIN-POINTS		PER CENT SUCCESSFUL
		Successful	Unsuccessful	
Cream	35	20	15	57.1
Whole milk	56	21	35	37.5
Skim milk	4	3	1	75.0
Ice cream	2	2	0	100 0
Totals	97	46	51	47 4

note the continued resistance of two of the cultures (nos. 8 and 15) to the fourth, fifth and sixth heatings. The organisms included in this study unquestionably resist pasteurization, but with the possible exception of culture no. 17, there is no evidence in these results which indicates that they would grow at pasteurizing temperature. If thermo-resistant types, similar to the organisms isolated in this work, should dominate the flora, the bacterial count would not be appreciably reduced by pasteurization. The average efficiency of pasteurization as reported in table 1 was only sixteen-hundredths of one per cent for the first pasteurization. If organisms of this type should become established in a pasteurizing vat, even its continued use throughout the day would not successfully destroy all of them.

High bacterial counts, on dairy products which have been

carefully pasteurized, may be readily accounted for by the presence of such thermo-tolerant organisms. That these organisms are comparatively common in dairy products is indicated in table 2, which shows that they were isolated from 57 per cent of the cream samples, from 37 per cent of the whole milk samples and from 47 per cent of the total number of samples tested.

The per cent of the flora which is made up of these heat resistant types is shown in table 3 which gives the average total bacterial count on sucrose agar of 12 samples of milk before and after each of three successive pasteurizations. It will be noted that on the average, "pin-points," capable of resisting two successive pasteurizations, constituted about 12.2 per cent of the original total count.

TABLE 3

*Per cent of "pin-points" found in twelve samples of milk after successive pasteurizations**

BEFORE PASTEURIZING, TOTAL COUNT PER CUBIC CENTIMETER	AFTER FIRST PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL	AFTER SECOND PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL	AFTER THIRD PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL
8,063,000	1,006,000	12.47	990,000	12.27	716,000	8.88

* Seven of these samples were pasteurized nine successive times. The reduction was marked after the fourth and fifth pasteurization, and only 0.37 per cent of the total count remained after the seventh pasteurization.

As previously pointed out, the pasteurizations in the foregoing experiments were conducted under laboratory conditions by means of flasks submerged in a water-bath. It was deemed advisable to determine whether the same results would be obtained in the pasteurization of larger volumes of milk in a vat under plant conditions. In coöperation with Prof. W. H. Martin of the Kansas Experiment Station, a 50-gallon rotary coil vat of skim milk was inoculated with several strains of pure cultures of "pin-points." After the inoculated milk was thoroughly agitated to insure equal distribution of the inoculum, about five gallons were drawn off into a sterile can for another part of the experiment. The remainder was sampled before and after each of

eight successive pasteurizations. The results are shown in table 4.

The 5-gallon portion of the inoculated milk previously mentioned was placed in a water bath and held at 63°C. for eight hours. Samples were taken each hour and plated immediately on sucrose agar. The results are shown in table 5.

An examination of the data in tables 4 and 5 shows that the cultures retained their thermo-resistant characteristics under vat conditions. More than 85 per cent of the organisms survived the first pasteurization, 28 per cent the second pasteurization, and 21

TABLE 4

Effect of eight successive vat pasteurizations of milk containing "pin-points"

Per cent of "pin points" surviving

FIRST PASTEURIZATION	SECOND PASTEURIZATION	THIRD PASTEURIZATION	FOURTH PASTEURIZATION	FIFTH PASTEURIZATION	SIXTH PASTEURIZATION	SEVENTH PASTEURIZATION	EIGHTH PASTEURIZATION
85.7	28.5	21.4	12.8	4.2	0.7	0	0

TABLE 5

The same milk used in table 4 held at pasteurizing temperature constantly for eight hours

Per cent surviving

FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR	SEVENTH HOUR	EIGHTH HOUR
17.4	5.28	1.4	0 1	0.01	0.0004	0	0

per cent were still alive after three pasteurizations, but after seven pasteurizations all were dead. Table 5 shows that some of the organisms were sufficiently heat resistant to withstand continuous exposure to 63°C. even for six hours.

It was hoped by these two experiments to simulate (1) the thermal conditions existing in a pasteurizing vat which is in constant use throughout the day, and (2) the thermal conditions of a continuous pasteurizer in eight hours use. The results in tables 4 and 5 emphasize the fact that these organisms will not grow under these conditions, although, their resistance to heat enables them to persist in either type of pasteurizing equipment for several

hours. The use of pasteurizing equipment, pipelines, separators, etc., for dairy products heavily seeded with thermo-resistant organisms, would necessitate thorough cleaning after each operation in order to prevent contamination of succeeding batches.

In an effort to find sources of pin-point colonies for further study, milk samples were subjected to various temperature conditions in the hope of stimulating the growth of these organisms. The results reported in table 6 further substantiate the conclusion that these organisms do not develop at pasteurizing temperatures. None of the plates from the samples before heating gave any evidence of pin-point colonies, therefore, the counts before heating represent the total counts without reference to any special kind of colony. The results after heating, however, represent the number of pin-point colonies and do not include any of the other thermophilic types which appeared on the plates. Table 6 includes only the samples from which pin-point colonies were isolated and the figures are averages of the results from all the samples involved. Successful isolations were not obtained in the case of the 17 samples held at 63°C. for twenty-four and forty-eight hours, but they are included in this table merely to show the effect of pasteurizing temperature. There were, however, many large surface colonies of thermophilic organisms on the plates

TABLE 6

Effect of holding milk at high temperatures on the development of pin-point colonies

NUMBER OF SAMPLES	TREATMENT OF THE MILK	AVERAGE NUMBER OF BACTERIA BEFORE TREATMENT	AVERAGE NUMBER OF "PIN-POINTS" AFTER TREATMENT	PER CENT OF THE ORIGINAL NUMBER
5	Held at 47°C.— 8 hours	3,420,000	40,240,000	1,176.0
3	Held at 47°C.—16 hours	226,500	1,260,000,000	556,291.0
3	Pasteurized three times then held at 47°C.—16 hours	25,230,000	19,673,000	78.0
3	Held at 56°C.— 8 hours	99,800,000	1,855,000	18.9
2	Held at 56°C.—16 hours	6,600,000	242,000	3.6
17	Held at 63°C.—24 hours	168,000	0	0
17	Held at 63°C.—48 hours	168,000	0	0

made from milk incubated at 63°C. Isolations from these colonies resembled the spore bearing rods described by previous investigators, but when cultivated at 37°C. they either refused to grow at all or continued to produce large, spreading, surface colonies. Throughout the progress of this work continuous effort was made to isolate types of organisms which produce pin-point colonies and grow in milk at the temperature of pasteurization. From nearly all samples of milk large colonies of thermophilic types were isolated but only under conditions of over-crowding of plates, could they be induced to produce small colonies.

The effect of different media on the growth of organisms which produce pin-point colonies

It has been suggested that the cause of pin-point colonies could be traced to the kind of medium which has been used since the war. Previous to the German blockade, Witte's peptone was very largely used in this country for the preparation of media. At that time also it was the common practice to adjust the reaction of media according to the Fuller scale. During and since the war, however, American peptones have come into greater favor for use in media, and the reaction of media is now almost exclusively adjusted according to the hydrogen-ion concept. The concurrence of these changes in methods with the agitation over pin-point colonies, together with data which they had collected, led Yates and Glover (1924) to suggest a causal relationship between the new methods of media preparation and the presence of small colonies in the plates.

Observations made in the Kansas Experiment Station laboratory suggested that at least some of the so called "pin-points" were saccharophilic organisms, and that some were even obligate in this respect. As pointed out earlier in this paper, it was noted that small colonies appeared in large numbers on low dilutions, but failed to appear in the expected numbers in the higher dilutions. The fact that the other colonies of normal size were present in the higher dilution plates in the expected numbers eliminated the probability of failure on the part of the technician

to make the proper dilution. It was further shown in preliminary experiments that if one cubic centimeter of a 1:100 dilution of sterile ice cream or milk were added to the higher dilution plates, the pin-point colonies would appear in the expected numbers.

In order to determine the effect of various kinds of media on the growth of the organisms which had been isolated, 42 cultures, selected at random from about 200 cultures, were plated on 14 different media. Four different kinds of plain agar were prepared as follows: (1) made with Difco peptone and the reaction adjusted to pH. 7.0, (2) the same batch of media except that the reaction established at Fuller's scale, +1, (3) made with Witte's peptone and the reaction established at pH 7.0, and (4) the same medium as no. 3, except the reaction was adjusted to Fuller's scale, +1. These four media are represented in table 7 under columns A, B, C, and D respectively. The analyses reported in columns Am, Bm, Cm, and Dm were obtained from plates to which 0.1 cc. of a 1:10 dilution (0.01 cc. of milk) of sterile milk was added in addition to media A, B, C or D, respectively. The media in A, and Am; B, and Bm; C, and Cm; D, and Dm; were, therefore, identical except that the plates in one case contained the same amount of milk as is found in a 1:100 dilution plate. The Fuller scale media used in this work had a reaction of pH 5.8. In table 7 it will be noted that for nine of the cultures, the milk-media were not used.

In addition to these eight media, simultaneous analyses were made on six other media, viz., glucose, lactose, sucrose, casein, milk powder and whey agars. The carbohydrate media (glucose, lactose, and sucrose) were from the same batch as medium A, except that one per cent of the carbohydrate was added in each case. Casein agar was prepared according to Ayers (1911), except that the reaction was adjusted to pH 7.0. milk powder agar was prepared according to Zoller (1913), and the whey agar according to Bouska and Brown (1921). For economy of space the individual counts on these fourteen media are not given, but the averages for the 42 cultures are included in table 7.

In table 7 is given the per cent of cultures which failed to grow,

TABLE 7

Effect of various kinds of plain agar and other media on the growth of pin-point colonies

	MEDIUM													
	A	Am	B	Bm	C	Cm	D	Dm	E	F	G	K	M	W
Composition of medium	Plain agar Difco pepton pH 7.0	Same as A, plus 0 01 of sterile milk per plate	Plain agar Difco pepton Fuller scale + 1	Same as B, plus 0.01 cc. of sterile milk per plate	Plain agar Wittes pepton pH 7.0	Same as C, plus 0.01 cc. of sterile milk per plate	Plain agar Wittes pepton Fuller scale + 1	Same as D, plus 0.01 cc. of sterile milk per plate	Glucose agar	Lactose agar	Sucrose agar	Casein agar	Milk powder agar	Whey agar
Number of cultures	42	33	42	33	42	33	42	33	42	42	42	42	42	40
Per cent of cultures showing no growth	38.1	3.0	73 8	45.5	50.0	30.3	42.9	42.4	0	0	0	28.6	0	25
Per cent of cultures showing less than half normal growth	16.7	12.1	2.4	3.0	14.3	3.0	11.9	6.1	2.4	0	0	2.4	2.4	7.5
Per cent of cultures showing normal growth	45.2	84.9	23.8	51.5	35.7	66.7	45.2	51.5	97 6	100	100	69.0	97.6	67.5
Average count on all cultures plated	10,400*	6,300	8,800	4,500	10,100	6,400	10,000	7,800	12,500	12,800	12,100	9,500	12,500	11,400

* "000" is omitted from the average.

the per cent which showed less than half normal development, and the per cent which found the medium favorable for their development. Each culture was plated on each of the different media from the same set of dilutions. The plates were incubated forty-eight hours at 37°C. and the colonies counted. All plates showing no growth were examined after forty-eight hours further incubation at room temperature before they were discarded. It will be noted that nearly all the cultures found the carbohydrate media (glucose, lactose, sucrose, and milk powder agar) favorable for their development. The normal growth for each culture was judged by the average number of colonies developing on the carbohydrate media. On some of the less favorable media the organisms either failed to grow at all, or grew only to a slight extent. For example, in some cases where 300 to 400 colonies were expected on a plate, in the less favorable media only 20 or 30 colonies developed. Although this could not be regarded as failure to grow, the small number of colonies, being outside the expected limits or error of the method, indicated a very unfavorable medium. In table 7 such cultures are tabulated as showing less than half normal growth; however, in most of these cases the growth was less than one-fourth and in many cases less than one-tenth the expected development.

A study of the individual counts together with the summary presented in table 7 brings out the following interesting facts.

1. Plain agar in general was unfavorable for the growth of "pin-points," 38 per cent of the cultures failed to grow on medium A, (Difco peptone, pH 7.0); 73 per cent refused to grow on medium B, (Difco peptone, Fuller scale +1); 50 per cent did not find medium C favorable (Witte's peptone, pH 7.0); and nearly 43 per cent of the cultures could not grow on medium D (Witte's peptone, Fuller scale +1).

2. The most unfavorable medium for the development of pin-point colonies was medium B, (Difco peptone, Fuller scale +1); only 23.8 per cent of the cultures made normal growth on this medium.

3. The addition of 0.01 cc. of milk to the Petri dish made it possible for many of the cultures which otherwise could not grow

to develop in the plain agar. Media Am, Bm, Cm, and Dm, supported 85, 51, 66, and 51 per cent of the cultures respectively, as compared to 45, 24, 35, and 45 per cent for the same media without the milk. Sixteen cultures failed to grow at all on medium A, but the addition of milk enabled fifteen of these to grow. The amount of milk added to these plates (0.1 cc. of a 1:10 dilution of sterile milk), is the same quantity of milk carried over from the sample in a 1:100 dilution. In this connection it should be pointed out that the dilution of the milk sample under analysis in all these experiments was in no case less than 1:10,000, so that the amount of milk carried over from the sample was not sufficient materially to affect the carbohydrate content of the medium.

4. The carbohydrate in the milk apparently is the constituent which is of benefit to the culture. This was proven by adding the same quantity of lactose (0.01 cc. of a 5 per cent solution) to plates as would be introduced with 0.01 cc. of milk; the substitution of lactose enabled cultures to develop which otherwise could not grow on plain agar. Addition of amounts of butter fat or casein, equivalent to that introduced in 0.01 cc. of milk, failed to supply the requirements for growth on plain agar.

5. All of the carbohydrate media (glucose, lactose, sucrose, and milk powder agar) were favorable for the growth of pin-point colonies. It may be noted that none of the cultures failed to grow on any of these media, and only 2.4 per cent (one culture), made less than half normal growth on glucose and milk powder agars.

6. The colonies on the carbohydrate media were slightly larger than on plain agar. This was especially true of milk powder agar on which the colonies in some cases were nearly twice as large as on plain agar, and usually larger than on the other carbohydrate media.

7. Casein agar, and whey agar were somewhat less favorable media for these organisms, although in general they were better than plain agar. Even in those cases where normal growth was obtained in casein agar, the colonies were frequently even smaller than they were on the plain agar media. The colonies on casein

agar in some cases were too small to be seen with a Leitz binocular stereo-magnifier, using 30× magnification.

8. Whey agar was suitable for the growth of only 67 per cent of the cultures. If the culture would grow at all on whey agar, the colonies were usually as large as on the other kinds of media.

It is quite evident from this table that if it is desired to isolate this type of organism, a carbohydrate medium should be selected. Experience has proved that milk powder agar is the most desirable, since it enables the colonies to become considerably larger. It is also evident that if it is desired to prevent organisms of this type from showing on the plate, plain agar with high acidity should be used.

Even with a relatively unfavorable medium, the cultures are likely to grow in the low dilutions (1:100). The small amount of milk introduced in a 1:100 dilution contained sufficient carbohydrate to enable 51 per cent of the cultures to develop on medium Bm, where as only 24 per cent could develop on the same medium without the milk. It is believed that this fact may account, in part, at least, for the supposed appearance of these organisms after pasteurization. Plates made on the raw milk are likely to be counted on high dilutions, which do not permit the development of all the pin-point colonies, even though the organisms may be in the agar plate. After pasteurization, lower dilution plates are observed for counting and the "pin-points" are detected. This explanation, however, would apply only for the saccharophilic type of "pin-points" which are under discussion.

Destruction of the organisms

The resistance of these organisms to steam and to hypochlorite disinfectants was determined in the following manner. About 2 liters of water were inoculated with 2 to 5 cc. of fresh milk cultures, from each of ten of the most resistant strains in the collection. The water was sampled and found to contain approximately 3 million "pin-points" per cubic centimeter. This water was poured into a series of sterile beakers, and after a few seconds was poured out. The beakers thus contaminated with the organisms were treated with steam or with chemical disinfectant

(B-K) for varying lengths of time, from thirty seconds to five minutes. After treatment, 100 cc. of sterile water was poured into the beaker and subsequently used for plating. The detailed counts are not given because they are only significant in showing the length of time necessary to kill all the organisms. It was found that one minute exposure to steam or the disinfectant used was sufficient to destroy all the organisms left in the beaker.

Biochemical and morphological studies

Sixty-five of the cultures isolated were selected at random and studied morphologically and biochemically. Each culture was inoculated into fermentation tubes containing one of the following: arabinose, starch, dulcitol, glucose, sucrose, glycerol, inulin, lactose, rhamnose, xylose and salicin. Two tubes of Clark and Lubs media were inoculated, one used to determine the Voges-Proskauer reaction, and the other used for the methyl-red test for acid production.

The cultures were also plated on carbohydrate agar in sufficiently high dilutions to give plates containing relatively few colonies; the colonies on these plates were measured, using the low power lens of a microscope equipped with a standardized filar micrometer.

The cultures were studied morphologically for size, shape, arrangement and motility, and also for their reaction to Gram's stain.

Preparation of media for fermentation tests

A modification of Enlow's synthetic sugar-free medium was used for the fermentation tubes. The composition of the medium was the same as given by Enlow (1923) except that the agar was omitted, thereby giving a liquid instead of a semi-solid medium. Brom-thymol-blue was added to the medium before sterilization (1.2 per cent of a 0.2 per cent alcoholic solution) according to the method devised by Baker (1922). This medium was standardized to a reaction of pH 7.2 before sterilization. The medium was placed in Durham fermentation tubes and sterilized in the autoclav. One per cent of the various sugar and other test

solutions was subsequently added aseptically. All fermentation tubes were incubated forty-eight hours at 37°C. before inoculation, and thirty days after inoculation.

The tubes were inoculated from a twenty-four-hour milk culture by means of a very small (1 mm.) platinum loop. In order to be sure that the amount of lactose carried over with the milk was not sufficient to permit the production of acid in the medium, a control tube of sugar-free medium was inoculated using the same loop. In no case was there any acid production or even growth in the control tube. The organisms were so strictly saccharophilic that they would not develop at all in the sugar-free medium, and, therefore, grew only in those tubes in which the sugar added could be utilized as a source of energy.

TABLE 8

Per cent of 55 cultures giving positive reactions to various carbohydrate fermentations and other tests

ARABINOSE	STARCH	DULCITOL	GLUCOSE	SUCROSE	GLYCEROL	INULIN	LACTOSE	RHAMNOSE	SALICIN	XYLOSE	VOGES-PROSKAUER	METHYL-RED	GELATIN LIQUEFACTION	MOTILITY
20	40	0	100	80	0	0	100	0	36	0	0	29	0	0

Results of fermentation studies

None of the 55 cultures fermented dulcitol, glycerol, inulin, rhamnose or xylose. None of the cultures produced gas in any of the fermentation tubes. All the cultures fermented glucose and lactose with the formation of acid. The percents of the cultures fermenting the other substances are given in table 8.

Colony study

The cultures used in the fermentation studies were also plated on carbohydrate agar, in order to study the size and shape of the colonies, in which case it was found that the small size of the colony was a constant character. The sizes of the colonies were measured by means of a filar micrometer standardized for a low power lens of a microscope.

Two distinct groups of colonies were recognized, which, for sake of convenience, will be called type A, and type B. Type A was a very small spindle shaped colony, too small to be seen with the naked eye, and barely visible with a hand lens. The size of the average was 0.150 to 0.175 mm. in the longest dimension. In dealing with these colonies, it was not infrequent to regard plates as sterile, which, on more careful examination, were found to contain 200 to 300 colonies. In the original isolation of the cultures used in this work, it was frequently difficult to decide whether or not some of the colonies isolated were small enough to be classed as "pin-points." The standards used in isolation were (1) that the organism should be at least thermo-tolerant, if not thermophilic, and (2) that it should characteristically produce a very small colony on carbohydrate media in thinly seeded plates. When small colonies were found which were slightly larger than typical pin-point colonies, yet sufficiently small to be doubtful, isolations were made, but in these cases the fact was recorded that the colony was atypical either in size or some other respect. Subsequent plating of these cultures proved that the slightly larger size of the colony was a constant characteristic of the organism. They were accordingly grouped together and called type B colonies. The size of the average was 0.250 to 0.350 mm. in the longest dimension of the spindle shaped colonies. Although these colonies were nearly twice as large as the type A colonies they were barely visible with the naked eye, but easily counted with the aid of a hand lens.

When the results of the fermentation studies were compared with the two types of colonies, A, and B, the following observations were made.

1. Those cultures which produced a very small colony, not visible with the naked eye, and difficult to see even with a hand lens (type A, about 0.150 to 0.175 mm.), were characterized by their inability to ferment salicin or arabinose, by their ability to ferment sucrose, and by the fact that they usually gave a methyl-red negative test.

2. Those cultures which produced a slightly larger colony, barely visible with the naked eye, and easily counted with a hand

lens (type B, size 0.250–0.350 mm.) were characterized by inability to ferment sucrose, by ability to ferment salicin or arabinose, and by a methyl-red positive test.

Each culture was examined under the hanging drop for motility, and all were found to be non-motile.

All the cultures were non-spore-bearing, Gram-positive rods, but varied considerably in size and arrangement. None were distinctly round coccus forms, although many resembled *Streptococcus lactis*, being very short rods or elongated spheres in pairs and short chains.

The cultures were divided into three morphological groups, the descriptions of which follow:

Group no. 1. Short rods, with pointed ends, mostly in pairs, and short chains, rarely exceeding four cells in length. The size of the average was 1.06 by 0.71 micron. This type resembled in appearance *Streptococcus lactis*, the common milk souring organism.

Group no. 2. One of the most characteristic morphological groups was a chain of irregular shaped rods. The chains consisted of from four to ten irregular shaped cells. One cell in the chain might be a distinct rod, adjacent to it might be a perfectly round cell, while adjacent to the spherical cell, perhaps, would be found a club shaped rod with one bulging and one tapering end. In another part of the chain one would likely find a pair of very regular, rod shaped cells, having a distinct diplo-bacillus arrangement, not at all like its clubbed or spherical neighbors. It was a most peculiar arrangement, suggesting the probability of involution forms resulting from unfavorable media or growth conditions. The arrangement, however, was quite consistent on all media capable of supporting growth. On carbohydrate broth, the chains were longer and slightly less irregular, but conforming to the above description.

Group no. 3. Another characteristic shape and arrangement noted in these cultures was a long, rather narrow rod, appearing in pairs, but one of the cells much shorter than its mate. It had the appearance of a terminal daughter cell, but if it were a daughter cell, no cases were observed in which the younger cell had grown so as to approximate the size of the mother cell. The size of the average was 1.95 by 0.78 microns.

Upon analysis of the data, it was found that the morphology of the organisms from type A colonies was distributed as follows: 42 per cent belonged to group no. 1, 42 per cent to group no. 2, 10 per cent to group no. 3 while the remaining 6 per cent did not conform to any of the groups and were dissimilar to each other. Of the type B colonies, 70 per cent belonged to the morphological group no. 1, 20 per cent to group no. 2, and the remaining ten per cent did not conform to any of the groups and were dissimilar to each other.

Disregarding the few cultures which did not conform to the three morphological groups it would seem that there are three kinds of organisms chiefly responsible for the pin-point colonies encountered in this study, each of which may be divided into two sub-groups.

1. Organisms resembling *Streptococcus lactis* morphologically: (a) Methyl-red negative, ferments sucrose, but does not ferment salicin or arabinose, produces a very small colony which is too small to be seen with the naked eye, but visible with a hand lens; (b) methyl-red positive, does not ferment sucrose, but ferments either salicin or arabinose, and produces a colony which is comparatively very small, but may be seen with the naked eye.

2. Organisms having a very irreglar morphology, appearing in chains of four to ten cells: (a) and (b) as described above.

3. Slender rod shaped cells appearing in pairs, with one cell much shorter than its mate: (a) and (b) as described above.

The fact that each of the three morphological groups is about equally divided among the subgroups (a) and (b) which produce the large and the small colonies respectively and that each of these subgroups gives the same general biochemical reactions, would indicate that there are two forms of each of the three kinds of organisms.

DISCUSSION AND SUMMARY OF RESULTS

In this paper an effort has been made to outline the pin-point colony problem and to study the bacteriological aspects of the causative organisms in at least one phase of that problem. It is

not contended that the organisms studied in connection with this investigation are the sole cause of pin-point colonies. Other workers have described spore-bearing rods, capable of developing at the temperature of pasteurization which have nothing in common with the types herein reported, except the size of the colony produced. It is entirely reasonable to assume that there are many organisms responsible for pin-point colonies which, as yet, have not been studied.

It was through a search for the thermophilic spore-bearing rods described by other workers that this investigation was started. Every sample of milk and cream studied was incubated under conditions which made possible the development of such types. Many and varied forms of thermophilic spore-bearing rods were isolated, but when cultured at 37°C., would not produce pin-point colonies. It is believed, however, that the organisms herein described do account for the pin-point colonies encountered in previous work in this laboratory, and may perhaps account for the conditions observed elsewhere.

The fact that these organisms develop only on low (1:100) dilution plates when plain agar is used, together with the fact that they are very difficult to detect, renders it very easy to miss them entirely in thickly seeded plates of raw milk or raw cream. On the other hand, after pasteurization the 1:100 dilution plates will very likely contain relatively fewer colonies of normal size, and the counting of such plates might reveal several thousand pin-point colonies. Several samples of milk from which pin-point colonies were isolated after pasteurization, did not show the presence of these types in the raw milk. The 1:100 dilution plates from raw milk in some cases were so thickly seeded as to mask the presence of the extremely minute types, and in other cases, where the bacterial count of the raw milk was very high, the low dilution plates were so thickly seeded that the growth of "pin-points" was completely inhibited. After pasteurization the larger colonies, which previously dominated the low dilution plates, had been killed and the "pin-points" were more easily detected.

Many times during this investigation high counts, due to pin-

point colonies, were obtained on pasteurized milk, and the "pin-points" were not detected in the routine analysis of the raw milk. The inference was that the organisms had developed during pasteurization. However, attempts to culture the organisms in milk at 63°C. repeatedly failed. Usually such cultures would survive one or two hours exposure at 63°C., but quantitative determinations indicated gradual diminution of numbers instead of growth. The conclusion was, therefore, drawn that the organisms responsible for the pin-point colonies encountered in this work were thermo-tolerant, but not thermophilic. Tanner (1924) emphasized the necessity of differentiating between thermo-tolerant and thermophilic bacteria. It is believed that some, though indeed not all, of the so-called increases in count after pasteurization may be explained on this basis. Those cases in which the pin-point colonies have definitely been proved to be the result of spore-bearing, thermophilic rods, capable of growing at 63°C., no doubt represent another phase of the problem.

In this work it has been demonstrated that in 47.4 per cent of the samples studied, thermo-tolerant, saccharophilic organisms were present which characteristically produced pin-point colonies. The resistance of these cultures to pasteurization is shown by the fact that, on the average, 99.84 per cent of the organisms survived the first pasteurization, 72 per cent the second, and 46 per cent the third. There was no conclusive evidence that any of these could grow during pasteurization, even though some of the samples were pasteurized seven times successively. The isolated cultures failed to develop at temperatures above 47°C.

A study of the effect of various kinds of media on the growth of these organisms showed several interesting observations as follows: Media containing a very small amount of carbohydrate greatly increased the chance of "pin-points" appearing on the plate. The small amount of lactose carried over in a 1:100 dilution of milk adds sufficient sugar to the medium to permit many of the types to develop that could not grow on higher dilution plates. Thirty-seven per cent of the isolated cultures grew normally on plain agar, but the addition of 0.01 cc. of

sterile milk to the plate enabled 64 per cent of the cultures to grow. Casein agar and whey agar were also somewhat unfavorable for the growth of "pin-points," but more favorable than plain agar. The standardization of the reaction of media to Fuller scale +1, frequently, though not consistently, resulted in a sufficiently acid medium to discourage the development of "pin-points." There was no consistent marked preference for Difco or Witte's peptone.

The most resistant pin-point cultures isolated were killed by heating in the steamer one minute or by treating for an equal time with a commercial hypochlorite disinfectant.

Differentiation of these thermo-tolerant types was made at the time of isolation on a basis of the size of the colony produced. Biochemical studies showed that the smaller type which were visible only with a hand lens (type A) were characterized by fermentation of sucrose and failure to ferment arabinose, or salicin, and a methyl-red negative reaction. Those types which were also thermo-tolerant but produced a colony large enough to be detected with the naked eye (type B), were characterized by ability to ferment arabinose or salicin, inability to ferment sucrose, and by a methyl-red positive test.

Morphologically all of the cultures were found to be non-motile, Gram-positive, non-spore-bearing, short rods or oval cocci resembling short rods. Although a few cultures were found which did not conform, most of the cultures were classified into three morphological groups: (1) very short oval rods or elongated cocci, resembling *Streptoccoccus lactis;* (2) short chains, four to ten cells in length, having very irregular morphology, and (3) short rod shaped cells in pairs one of the cells being much shorter than its mate.

If the organisms are resistant to pasteurization, their subsequent destruction in milk is hopeless, and the solution of the "pin-point" problem must be through preventive measures. Some have suggested that a new medium be devised which would not permit the development of pin-point colonies; others have implied that their presence in the plate should simply be ignored. It should be borne in mind that the premises on which

bacterial counts are based are, that the conditions which make possible the entrance and development of large numbers of bacteria are not ideal conditions for the production and handling of such a delicate food. If milk contains large numbers of bacteria, regardless of whether they are "pin-points" or not, it indicates that the milk has not received proper attention. Deliberately to overlook the presence of certain types would be to ignore the undesirable conditions responsible for their presence.

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Charles Edward Marshall (1866–1927)

President, Society of American Bacteriologists, 1914

Charles Edward Marshall

1866-1927

A great institution is the lengthening shadow of a single man. The Laboratory of Bacteriology and Hygiene at Michigan State College is the lengthening shadow of Charles Edward Marshall, who was directly and almost solely responsible for the development of these subjects as early as 1902 at a small, but old and conservative midwestern land-grant college. It is noteworthy that in spite of the urgent demand for a building to house physics, the oldest and most substantial of sciences—with all the prestige of this great discipline and with its obvious need on the campus—Professor Marshall succeeded in his efforts to have adequate provision made for what was, at that time, the youngest of the sciences. Justification of his noteworthy efforts and of his success need not be a matter of discussion at this time. There are many who are willing to testify, if not to the wisdom of the choice between bacteriology and physics, at least to the notable services which the laboratory, that was erected under Marshall's direction rendered to the State and to humanity while operating under his wise direction.

Professor Charles Edward Marshall was born October 6, 1866. He received his degree of Bachelor of Philosophy from the University of Michigan in 1896. He studied in Jörgensen's laboratory at Copenhagen in 1898 and received his Ph.D. from Michigan in 1902. Returning to Europe he studied in the Pasteur Institute in 1903 and again in 1913. In the meantime he became assistant bacteriologist at the University of Michigan, and then bacteriologist to the Michigan Agricultural Experiment Station at Michigan State College. He was appointed professor of Bacteriology and Hygiene in 1902 and scientific and assistant director of the Experiment Station in 1908.

In 1912 he was called to the position of the Directorship of the

Graduate School and Professorship of Microbiology at the Massachusetts Agricultural College in which capacity he served until his death, March 20, 1927. At the Massachusetts institution he was again notably successful in having adequate material equipment made available for microbiological teaching and research. The Massachusetts Collegian said, at the time of his death, "As a result of Dr. Marshall's efficient management and his high ideals the Graduate School has developed from an ill-organized and rather purposeless organization of two or three students to a strong department of the college with about fifty students at the present time."

He distinguished himself as the editor of the most comprehensive textbook in our language on microbiology, as a research worker in his chosen field, and as a director of the research activities of others. But, perhaps especially as a teacher who had a big view of a new subject, Dr. Marshall is deserving of a distinguished place. His most notable research was on milk, with special reference to the associative action of microbes.

He was a man to whom his friends were drawn as by a magnet and an opponent whom one found decidedly stimulating. He was the friend of the young man and woman who aspired to achievements in scientific pursuits. His interest in the Society of American Bacteriologists dated from its inception. He served most faithfully and fruitfully as the Society's secretary and with distinction as its President (in 1914).

It is doubtful if any one of his generation had a better comprehension of the field of bacteriology than Dr. Marshall. This is exemplified by a statement made to me, a microbiological neophyte, over twenty years ago by a distinguished botanist at a Southern college: "Marshall has covered the earth like a tent with his outline for the study of microbiology."

WARD GILTNER.

INTERFACIAL TENSION AND BACTERIAL GROWTH

NEAL DAVIS

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The object of this article is to point out the parallelism which exists between the effect of cations, on interfacial tension and on bacterial growth. Previous work has been done by Larson, Cantwell and others in which they have shown that soap solutions lower the tension of the film between the surface of a culture medium and the air; and they have also indicated that there is a toxic effect which accompanies this lowering of the surface tension. Recent investigation by Gibbs, Batchelor and Sickels, has shown that the toxic effect suggested by Larson may be due to the nature of the material used to lower the surface tension.

The author of this article has recognized that measurements made of the film at the surface of the medium may not express the true relations which exist at the film between the oil capsule of the bacterium and the medium. Therefore, an attempt was made to devise methods and design apparatus to make this measurement. Several attempts were made, each exposing its own mistakes and suggesting new possibilities, until the present method was adopted as the result of the following reasoning.

The impossibility of isolating a bacterium and measuring the tension at its surface led the writer to try to approximate the conditions which must exist in a state in which it could be measured. Since a bacterium is surrounded by an oil capsule which must enlarge as the bacterium grows, the conditions at the surface of its capsule must be similar to the conditions of the film of any drop of oil which is increasing in size while surrounded by a liquid. If a jet of oil is sprayed into water it breaks up into drops the size of which depends upon the interfacial tension between the oil and water. If the drops are made to form very slowly and their volumes accurately measured, a method results

for a comparison of the tension developed between a given oil and various solutions. Those solutions which affect the size of the oil drops were expected to have a similar effect on growing bacteria.

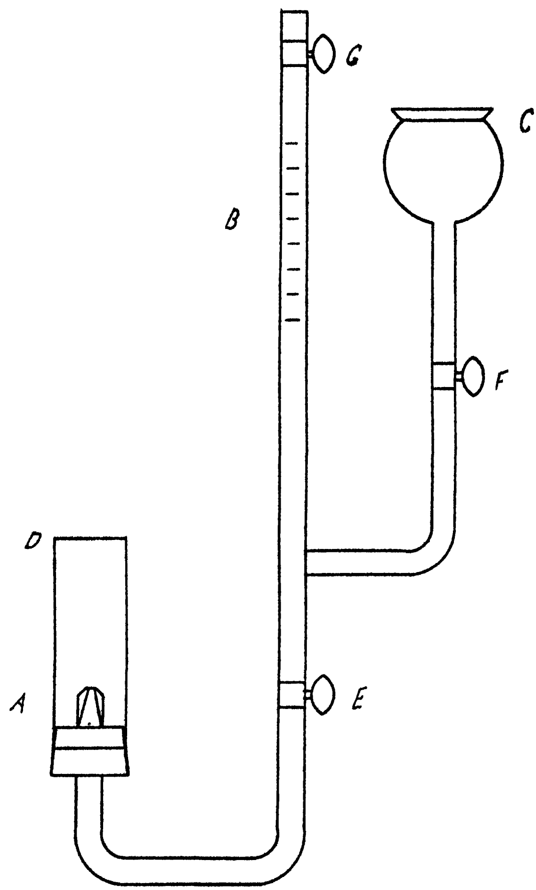

FIG. 1

APPARATUS

The apparatus shown in the accompanying figure was constructed. The aperture of the tip *A* has a diameter of 0.4 mm. The face was ground to a plane and polished with fire. The arm

B is a capillary tube of diameter 2.5 mm., and graduated in 0.01 cc. Oil is introduced at *C*. After the "interfaciometer" is filled and all air bubbles removed, the solution to be tested is introduced into *D*. The apparatus is then immersed in a constant temperature bath, 37.5°C. When the solution in *D* reaches a constant temperature, the meniscus in the arm *B* is drawn up to the zero level. Then, with stopcocks *E* and *G* open and *F* closed, thirty drops of oil are allowed to form at *A*. As soon as the last drop breaks off, *E* is closed and the volume of the thirty drops

TABLE 1

Effect of cations upon size of oil drops		Effect of cations upon bacterial growth	
Cation	Volume in cubic centimeter 30 drops	Cation	Molar concentration inhibiting growth
Water	0.0166		
Na^+	0.0163	Na^+	1.5
NH_4^+	0.0160	NH_4^+	1.0
K^+	0.0130	K^+	0.8
Ni^{++}	0.0116	Ca^{++}	0.75
Mn^{++}	0.0113	Sr^{++}	0.40
Al^{+++}	0.0113	Mn^{++}	0.04
Ca^{++}	0.0106	Ni^{++}	0.006
Fe^{+++}	0.0098	Al^{+++}	0.004
Sr^{++}	0.0091	Fe^{+++}	0.004
Cu^{++}	0.0090	Cu^{++}	0.002
Cd^{++}	0.0076	Cd^{++}	0.0002
Hg^{++}	0.0050	Hg^{++}	0.00002

read directly from the graduations on *B*. The tip at *A* must be thoroughly cleaned before each determination and the work repeated until constant readings are obtained.

SELECTION OF THE OIL

The physical properties of closely related homologues within an organic series differ but little, and since the oil capsule of a bacterium is thought to be an ester of a high fatty acid, it was held that any high fatty acid might be used if it had the following physical characteristics,—insoluble in water, a liquid at incubation temperature, low density, comparatively low viscosity and

obtainable in pure form. Oleic acid, Bakers U. S. P., answered these conditions and was used.

The chlorides were chosen because they have comparatively little effect on organic materials and offer a large choice of soluble salts. Those chlorides such as zinc and barium which present a cloudy solution could not be used since bacterial growth was to be judged, later, by the appearance of a cloud. In all cases a one molar solution was used except with mercury, where the solubility would not permit this concentration. Cadmium was measured in various concentrations and the volume plotted against concentration. Similar measurements were made for mercury up to ½ molar and the curve drawn similar to that for cadmium to approximate the one molar reading. The first half of the table gives the volumes of the oil drops in the various solutions, thus indicating the effect of the cation on the interfacial tension. To show that the effects were due to the cations and not to variation of hydrogen ion contents, HCl was added to the less acid solutions until by colorimetric titration, all solutions showed the same pH value. Tests were made on these solutions and the same results were obtained.

With this series at hand, it was desired to know if bacteria would react with these solutions and yield a similar series. If the force causing the oil drop to enlarge becomes small, or if the interfacial tension increases sufficiently, the oil drop can not increase in size. Likewise, if the expanding power of the bacterium is small, or the interfacial tension about it increases, the bacterium cannot grow. With this reasoning as a basis, the following experiments were tried.

General growth characteristics were desired and not cultural characteristics, therefore a mixed culture was used. A 1 per cent solution of Bacto-Pepton was made from distilled water and used as a culture medium. One hundred cubic centimeters were inoculated with *B. coli* and 5 cc. of tap water. After forty-eight hours of incubation at 37.5 cc. an inoculation was made into a sterile tube, and this was incubated for forty-eight hours and used for a stock culture. A series of culture tubes was arranged, each containing 10 cc. of culture medium and each was

inoculated with a loop from the stock culture. To the respective tubes were added varying quantities of the solutions of the cations. The quantities of the solutions of the cations were increased until all signs of turbidity caused by bacterial growth, were prevented for forty-eight hours. When additions of the cations became large enough to change appreciably the concentration of the medium, equivalent quantities of the dry salts were weighed out and dissolved in the medium.

The cations again arrange themselves in a series as shown in the second half of the table. The figures give the molar concentration of the cations necessary to prevent the appearance of turbidity for forty-eight hours of incubation. Again the pH concentration was equalized in all the tubes without a change in results.

Hotchkiss in studying the effect of the cations on *B. coli*, obtained a similar series, but attributed it to toxic effects. To see whether or not the bacteria were actually poisoned, the writer made up a solution of cadmium, 0.0003 molar, which prevented growth indefinitely. Inoculation was made as usual into this medium and incubation continued for seven days with no growth. At the end of this period the culture was concentrated in a centrifugal machine and a sterile tube inoculated from it and incubated with a check. At the end of forty-eight hours a cloud appeared in the inoculated tube while the check was clear. This showed that the bacteria were not killed, but were merely prevented from growing by some force. It is evident that if the cation does increase the interfacial tension, there should be a concentration which would just grip the bacterium tight enough to prevent growth and yet not do it any permanent harm. Hotchkiss also noticed a stimulating effect due to the cations in weak solutions. This was noticed with cations of great toxic powers as well as with others. But if the bacterium in the process of fission is looked upon as an oil drop pinching in two in the center, it can readily be seen how a slight increase in interfacial tension would tend to stimulate the process, while a considerable increase would prevent it.

A comparison of the two series shows a definite parallelism.

It thus becomes evident that if the condition at the surface of the bacterium has been approximated there must be a definite relation between interfacial tension and bacterial growth. Inconsistencies in the series are expected because the results were determined for only a few bacteria and only one oil was used, which may not be entirely characteristic. Such factors as dissociation and adsorption introduce variables. Some of the cations would be expected to have a stimulating effect on metabolism while others would have a real toxic effect. It is recognized that there is such a thing as specific toxicity; but, these results seem to indicate that a failure to grow may be due to mechanical force as well as to toxicity.

In concluding, the author wishes to acknowledge the interest of Wm. C. Beaver, of the department of Biology of Wittenberg College, and also the helpful suggestions given by J. E. Taylor, of the department of Chemistry. Mr. Taylor also gave assistance in the construction of apparatus.

SUMMARY

A method has been devised for the measure of interfacial tension in a condition which approximates that of the film between the oil capsule of a bacterium and the culture medium.

The interfacial tension was measured between oleic acid and molar solutions of 12 chlorides, and the results arranged in a series.

A determination was made of the molar concentration of the chlorides in pepton broth, which would just prevent bacterial growth, as indicated by the appearance of a visible cloudiness for forty-eight hours at 37.5°C. The results were arranged in a series which showed a marked parallelism with the first series.

If bacteria are looked upon as analogous to oil drops, both the stimulating and the toxic effects of cations can be explained.

THE RELATION OF SURFACE TENSION TO BACTERIAL DEVELOPMENT

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INTRODUCTION

Considerable attention has been directed to those environmental factors which affect the growth and activities of bacteria. Factors related to the culture medium, such as reaction, concentration of the different constituents, methods of sterilization, oxygen tension, and vitamines, have been thoroughly investigated. Studies along these lines have made it possible to cultivate organisms that hitherto have refused to develop on ordinary artificial media. Some of the physical factors such as temperatures and osmotic pressures have also been included in the research program. One physical factor, however, which has received little attention is the relation of surface tension to bacterial development.

HISTORICAL REVIEW

Historical notes on this subject have been cited in a previous paper published from this department, Gibbs, Batchelor and Sickels, 1926. There are, however, several recent contributions which deserve specific mention.

Albus and Holm (1925) found that *Lactobacillus acidophilus* grew very well in a medium with a surface tension as low as 36 dynes per centimeter while *L. bulgaricus* in the same medium depressed to a surface tension of 40 dynes showed no growth after seven days incubation at 37°C. They proposed this as a

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means of differentiating between these two closely related organisms and concluded that the surface tension might be a factor in the implantation of these organisms. They did not report the method employed in the determination of the surface tension, the chemical nature of the medium, or the substances used as depressants.

Marshall (1924) studied the surface tension of mediums by methods which he terms "static" and "dynamic," or the drop, and the ring, methods. He used the stalagmometer for the dynamic measurements and the tensiometer for the static. He performed various experiments with nine standard mediums including broth, agar, bile, and gelatin in non-solidifying concentrations. He found that various inorganic and organic substances, exclusive of soap solutions, oils and the like, had little effect on the surface tension of infusion broth. In the experiments on aqueous solutions, he found that very small amounts of peptone, beef extract, ascitic fluid, castor oil soap, and infusion broth brought about a marked reduction of surface tension. This was not the case, however, in glucose and sodium chloride solutions. A comparison of static and dynamic measurements gave radically different values for the surface tension. Marshall concluded that the surface tension of infusion broth or of a synthetic medium is not affected as a consequence of bacterial growth; that organisms growing in mediums of different surface tension appear to have different rate of growth, and different gas metabolism; and that correlation of these variables reveals no systematic variation.

Marshall and Robinson (1925) studied the relation of surface tension to diphtheria toxin production. In their studies they attempted to correlate the pellicle formation of the organism and the surface tension of the medium. They found that the surface tension of different lots of veal infusion toxin broth varied usually from 45 to 47 dynes per centimeter. The surface tension could not be raised by any practical means but could be lowered as far as 35 dynes per centimeter by the addition of minute amounts of castor oil soap. They found further that toxin broth of a surface tension of 40.9 dynes per centimeter obtained

with soap solution gave better pellicle formation than broths having a higher or lower surface tension, and that broths having a surface tension as low as 35 dynes per centimeter inhibited pellicle formation completely and usually prevented growth of the organism.

Frobisher (1926) concluded that surface tension is an important factor in bacterial growth, in morphology, and probably in cell division; and to a less extent in the motility of bacteria and their ability to retain Gram's stain. He also found that low surface tension is probably an important factor in determining the size of the individual cells, the rate and extent of cell division, and the cell grouping, and that the loss of virulence of pneumococci after treatment with surface tension reducents may be due to the disintegration of these bacteria when they are subjected to low surface tension. He attributes the dissolution of pneumococci by bile to the low surface tension induced by this substance. This investigator found that the serologic types of pneumococci do not differ from one another in their sensitiveness to the low surface tensions. He also found that sodium oleate is the most useful reagent for reducing surface tension of culture mediums. He suggests the differences in ability of organisms to grow at low surface tensions as a means of differentiation between species of bacteria, and that Huntoon's "hormone" medium for pneumococci may be improved by raising the surface tension with charcoal to some point above 54 dynes per centimeter.

GENERAL METHODS

In the present study, all surface tension measurements unless specifically noted were made both by the method of Fahrenwald (1922) and by the drop weight method. The depressants consisted of four soaps prepared from castor oil, cocoanut oil, olive oil, and palmitic acid. These soaps were the same which were used in the experiments described in the previous report. Two per cent stock solutions of each soap were prepared and added by pipette in the desired amount. The soap additions in the data which follow are expressed in per cent of dry soap. The medium consisted of glucose beef infusion broth prepared as follows: 10

grams of bacto-pepton "difco" were added to 1000 cc. of beef infusion. These were boiled together in the Arnold sterilizer for two hours and filtered. To the unsterile broth, glucose was added in the proportion of 1 gram per 100 cc. When the sugar was dissolved, the solution was divided into flasks, the necessary soaps added, and sterilization performed in the autoclave at 10 pounds pressure for twenty minutes. Sodium chloride was omitted, fearing that it might form precipitates with the depressants.

EXPERIMENTAL DATA

Determination of the surface tension of broth

It is a fact commonly understood that surface tension depressants contained in a solution have a tendency to accumulate at the surface. Two experiments were therefore planned to determine the effect of such concentration of depressants on the surface tension as determined by the film method.

The medium employed was infusion broth containing 1.0 per cent glucose. In one series of the tests the broth was thoroughly stirred immediately preceding each measurement. The broth was poured into the measuring container and the surface tension immediately determined. A second reading was made, then, a third, fourth, fifth, etc., until four successive readings gave constant values. The medium was then allowed to remain in the container for ten minutes, after which time it was thoroughly stirred and the measurements repeated. In a second series of tests the above procedure was repeated except that the broth was not stirred before making surface tension measurements. The results of these two series of tests are shown in tables 1a and 1b.

It is seen from the data in tables 1a and 1b that a constant value for the surface tension is obtained, usually at the second reading, when the solution is stirred immediately before taking the measurement while from three to nine measurements are necessary to reach a constant when the solution is not stirred. It will also be noted that the surface tension constant when obtained after stirring the medium was 42.3 dynes per centimeter while the constant obtained for the same medium without stirring

was 39.6 dynes. The higher constant in the former case is no doubt due to the more uniform dispersion of the depressant,

TABLE 1A

Surface tension measurements of broth which was thoroughly mixed before making the determination

Surface tension measurements dynes per centimeter

TIME	1	2	3	4	5	6	7
0	42.4	42.3	42.4	42.3	42.3	42.3	42.3
10 minutes	42.6	42.3	42.3	42.3	42.3		
30 minutes	42.4	42.3	42.4	42.3	42.3	42.3	42.3
1 hour	42.3	42.3	42.3	42.3			
2 hours	42.4	42.3	42.3	42.3	42.3		
4 hours	42.6	42.4	42.3	42.3	42.3	42.3	
5 hours	43.0	42.6	42.3	42.3	42.3	42.3	
6 hours	42.6	42.3	42.4	42.3	42.3	42.3	42.3
7 hours	42.4	42.3	42.3	42.3	42.3		
8 hours	42.6	42.3	42.3	42.3	42.3		
12 hours	42.6	42.3	42.3	42.3	42.3		

TABLE 1B

Surface tension measurements of broth undisturbed

Surface tension measurements dynes per centimeter

TIME	1	2	3	4	5	6	7	8	9	10
0	42.4	42.2	42.2	40.8	40.2	40.0	39.6	39.6	39.6	39.6
10 minutes	42.0	42.0	39.8	39.6	39 6	39.6	39.6			
30 minutes	41.6	41.3	40.2	39.8	39.6	39.6	39.6	39.6		
1 hour	41.0	41.0	40.3	40.3	40.3	40.0	40.0	40.0	39.6	39.6
2 hours	40.6	40.4	39.8	39.8	39.6	39.6	39.6	39.6	39.6	39.6
4 hours	40.3	40.1	39.6	39.6	39.6	39.6	39.6	39.6		
5 hours	39.8	39.8	39.6	39.6	39.6					
6 hours*	39.6	39.6	39.6	39.6						
7 hours	40.4	39.8	39.6	39.6	39.6	39.6				
8 hours	40.8	39.8	39.6	39.6	39.6	39.6				
8 hours with stirring	42.6	42.3	42.4	42.3	42.3					
12 hours	41.2	40.0	39.9	39.6	39.6	39.6	39.6			

* At six hours the plate was immersed fifteen minutes before the surface tension was measured.

while in the latter case the depressants were more concentrated at the surface.

Two unusually interesting points are brought out in the data of table 1b. It will be noted that at the six-hour interval the silver disc was allowed to remain immersed in the broth for a period of fifteen minutes before the surface tension was measured. The first measurement then gave the constant indicated in table 1b. The second interesting point was at the eight-hour period. In this case the medium was stirred before measurement and gave at the second reading the same constant found in table 1a.

The results of these two experiments show that there are two distinct methods of applying the film method to surface tension

TABLE 2

Surface tension of broth containing soaps

	Soaps					
	Castor			Olive		
Percent	0.002	0.01	0.02	0.02	0.06	0.1
Film method (dynes /cm.)	43.1	36.1	34.0	44.5	37.3	36.3
Drop weight method	56.1	53.3	53.0	60.5	56.6	53.4

	Soaps						
	Cocoanut			Palmitic			Control
Percent	0.02	0.06	0.10	0.02	0.06	0.10	
Film method (dynes/cm.)	38.3	35.0	33.8	40.2	33.0	27.1	48.3
Drop weight method	57.1	53.2	52.8	57.7	53.4	41.6	60.8

measurements. By agitation of the liquid, a constant is easily obtained which is the result of the complete dispersion of the depressants, while a lower constant may be obtained by allowing the plate to remain immersed in the liquid for a period of fifteen minutes or by not disturbing the liquid before making the measurement. Which of these methods is preferable or more accurately represents the actual condition of the medium during the period of bacterial growth is not possible to determine. The measurements reported in the following text were made after stirring the medium to a point of equilibrium.

Determination of surface tension of broth containing depressants

Various concentrations of the depressants were added to glucose broth and the surface tension determined. The results are shown in table 2.

It will be noted from the data in table 2 that considerable variations of surface tension were obtained by the soap additions, not only in the actual depression but in the proportion of soap necessary to produce it. On the basis of the amount of soap necessary to produce a given surface tension, castor oil soap is the most efficient, 0.002 per cent giving a depression of about 5 dynes below the control and 0.02 per cent giving about 14 dynes depression. Palmitic soap is slightly less efficient and olive soap is about the same. Cocoanut oil soap produces the lowest surface tension among the four depressants but is in general not as satisfactory on account of its turbidity and tendency to form precipitates.

Determination of the toxicity of the soaps

The foregoing experiments proved the value of the four depressants in respect to their ability to lower the surface tension without alteration of hydrogen-ion concentration. Since the toxicity of the depressants reported by other investigators has always been a variable and unknown quantity, it was thought advisable to determine, if possible, the toxicity of the soaps prepared for these experiments.

Broths were therefore prepared which contained 0.01 per cent, 0.02 per cent and 0.06 per cent concentration of the different soaps. These were tubed in approximately 5 cc. lots and sterilized at ten pounds pressure for twenty minutes. A tube of each concentration was then inoculated with a twenty-four-hour culture of each of the following organisms: *Diplococcus pneumoniae*, *Streptococcus viridans*, and *Streptococcus hemolyticus*. The cultures were incubated at 37°C. and observations made after one, two, three and five days. The pH was used as the index of the activity of the organisms.

The results are shown in tables 3a, 3b, and 3c. It will be seen

from the data in tables 3a, 3b, and 3c, that growth occurred in all concentrations of olive, cocoanut, and palmitic soaps after the first day of incubation. Castor soap, however, seems to be toxic to the organisms. In the concentration of 0.01 per cent castor soap only *Streptococcus viridans* and *Streptococcus hemolyticus* developed and in these cases growth was not as vigorous

TABLE 3A

Determination of toxicity of the soaps

Diplococcus pneumoniae

	TREATMENT					
	Castor			Olive		
Concentration	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%
Surface tension (dynes/cm.)	44.2	41.0	39.0	45.4	43.6	40.2
Age of culture:	pH	pH	pH	pH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2
1 day	—*	—	—	5.1	5.0	5.1
2 days	5.8	—	—	5.0	5.0	5.0
3 days	5.6	—	—	4.8	5.0	5.0
5 days	5.2	—	—	4.6	4.8	5.0

	TREATMENT						
	Cocoanut			Palmitic			Control
Concentration	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%	—
Surface tension (dynes/cm.)	44.3	39.0	36.1	39.6	37.3	34.9	49.8
Age of culture:	pH	pH	pH	pH	pH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2	7.2
1 day	5.6	5.8	6.0	5.0	5.0	5.2	4.8
2 days	5.4	5.7	6.0	4.5	4.9	5.0	4.7
3 days	5.4	5.7	6.0	4.5	4.6	5.0	4.6
5 days	5.4	5.7	5.8	4.5	4.6	4.8	4.4

* — No growth.

as in the other tubes. *Diplococcus pneumoniae* failed to develop in any of the concentrations until reinoculated and then failed to develop in concentrations of 0.02 per cent and 0.06 per cent. After reinoculation and two days incubation, *Diplococcus pneumoniae* grew in the tube containing 0.01 per cent castor soap but growth was somewhat retarded. On reinoculation, *Streptococcus viridans* and *Streptococcus hemolyticus* developed in the concentra-

tion of 0.02 per cent but growth was not vigorous. Subsequent inoculations of the tubes gave no further development.

Cocoanut soap showed slight toxicity in all concentrations used although the most marked effect was in the 0.06 per cent concentration. The concentrations used of palmitic and olive oil soaps showed no toxic effects. It will be noted that the lack of de-

TABLE 3B

Determination of toxicity of the soaps

Streptococcus hemolyticus

	TREATMENT					
	Castor			Olive		
Concentration	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%
Surface tension (dynes/cm.)	44 2	41.0	39.0	45.4	43.6	40.2
Age of culture:	pH	pH	pH	pH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2
1 day	6.2	—*	—	5.0	5.0	5.1
2 days	5.0	—	—	4.7	4.8	5.0
3 days	5.0	—	—	4.6	4.6	4.6
5 days	5.0	—	—	4.6	4.6	4.6

	TREATMENT						
	Cocoanut			Palmitic			Control
Concentration	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%	—
Surface tension (dynes/cm.)	44.3	39.0	36.1	39.6	37.3	34.9	49.8
Age of culture:	pH	pH	pH	pH	oH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2	7.2
1 day	5.6	6.0	6.4	4.8	4.8	4.8	4.6
2 days	5.4	5.8	6.2	4.6	4.8	4.8	4.5
3 days	5.4	5.6	6.2	4.5	4.7	4.8	4.5
5 days	5.4	5.6	6.2	4.5	4.7	4.7	4.5

* —No growth.

velopment of the organisms in the castor soap series was due to toxicity and not to the surface tension depression. This is made clear by the palmitic series which has a lower surface tension than the castor series and at the same time better growth.

These results show that castor oil soap in the concentration of 0.01 per cent is toxic to *Diplococcus pneumoniae* while concentrations of 0.02 per cent and 0.06 per cent are very toxic to *Diplo-*

coccus pneumoniae, Streptococcus viridans, and *Streptococcus hemolyticus.*

These results are of interest because they point out the many difficulties which would have been encountered in later experiments had these concentrations been employed. The concentrations used in the experiments which follow were castor 0.006

TABLE 3c

Determination of toxicity of the soaps

Streptococcus viridans

	TREATMENT					
	Castor			Olive		
Concentraction	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%
Surface tension (dynes/cm.)	44.2	41.0	39.0	45.4	43.6	40.2
Age of culture:	pH	pH	pH	pH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2
1 day	6.0	—*	—	4.7	5.2	5.1
2 days	5.1	5.4	—	4.7	4.7	4.8
3 days	4.8	5.2	—	4.6	4.6	4.7
5 days	4.6	5.0	—	4.6	4.6	4.6

	TREATMENT						
	Cocoanut			Palmitic			Control
Concentration	0.01%	0.02%	0.06%	0.01%	0.02%	0.06%	—
Surface tension (dynes/cm.)	44.3	39.0	36.1	39.6	37.3	34.9	49.8
Age of culture:	pH	pH	pH	pH	pH	pH	pH
0 day	7.2	7.2	7.2	7.2	7.2	7.2	7.2
1 day	5.5	6.0	6.2	4.7	4.7	4.7	4.7
2 days	5.4	6.0	6.0	4.7	4.7	4.7	4.7
3 days	5.5	5.6	6.0	4.6	4.7	4.7	4.5
5 days	5.4	5.6	5.6	4.6	4.6	4.6	4.5

* —No growth.

per cent, olive 0.06 per cent, cocoanut 0.06 per cent, and palmitic 0.06 per cent.

Surface tension and the growth of Diplococcus pneumoniae

One per cent glucose broth to which were added the different soaps in their desired concentrations was flasked in 60 cc. lots, sterilized at ten pounds pressure for twenty minutes, and in-

TABLE 4

Diplococcus pneumoniae and surface tension

	TREATMENT																			
	Castor 0.006%				Olive 0.06%				Cocoanut 0.06%				Palmitic 0.06%				Control			
	Culture		Control		Culture		Control		Culture		Control		Culture		Control		Culture		Control	
	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH
Film method																				
Age of culture:																				
0 day			44.8	7.0			40.2	7.0			39.6	7.0			40.7	7.0			53.0	7.0
1 day	40.0	5.8	44.4	7.0	38.6	5.1	39.8	7.0	36.8	5.6	39.0	7.0	38.3	4.9	39.9	7.0	48.0	4.8	52.5	7.0
2 days	43.3	5.8	43.4	7.0	38.7	5.0	39 4	7.0	38.4	5 6	40.2	7.0	38.4	4.8	40.0	7.0	48.6	4.8	52.4	7.0
3 days	43.8	5.8	44.4	7.0	39.0	5.0	39 8	7.0	39.8	5.6	40.8	7.0	39.2	4.7	39.8	7.0	50.0	4.5	52.4	7.0
5 days	44.0	5.8	44.0	7.0	39.7	5.0	39.7	7.0	40.7	5 6	40 0	7.0	39.7	4.7	39.8	7.0	50.0	4.5	53.3	7.0
7 days	42.8	5.8	47.6	7.0	38.8	5.0	40 1	7 0	38.1	5.6	39.2	7.0	38.7	4.7	42.3	7.0	48.3	4.5	54.8	7.0
10 days	42.3	5.8	46.6	7.0	37.6	5.8	41.9	7.0	38.0	5.6	41.7	7.0	37.4	4.6	44.5	7.0	48.5	4.5	55.2	7.0
Drop weight method																				
Age of culture:																				
0 day			53.3				49.3				49.6				49.8				60.0	
1 day	48.6		50.0		48.6		48.7		47.8		47.8		46.3		48.5		57.3		62.0	
2 days	49.7		52.2		49.0		49.4		50.2		49.3		49.7		50.9		60.0		62.0	
3 days	49 7		52.3		49.5		49.0		49.3		51.2		50.2		51.7		57.9		61.7	
5 days	48.4		50.4		49.2		51.2		49.6		50.4		49.8		50.2		59.8		60.9	
7 days	51.6		52.2		48.7		51.6		46.3		50.3		47.6		49.8		54.6		61.3	
10 days	48.9		53.4		46.2		52.0		45.2		51.7		46.2		52.2		52.9		62.2	

oculated with a twenty-four-hour culture of *Diplococcus pneumoniae*. Twelve flasks for each concentration including the control were prepared, six of which were inoculated and six uninoculated. The cultures together with the controls were incubated at 37°C. and observed after one, two, three, five, seven, and ten days. This was done in order to make possible the observation of any phenomenon that might occur at some definite surface tension during the course of the experiment. A systematic arrangement of the flasks and the results obtained are shown in table 4.

The data in table 4 reveal a certain relation between the surface tension as determined by the film and the drop weight methods. In spite of the fact that the drop weight method gives much higher values than the film method, two curves that are almost parallel are obtained from the surface tension measurements recorded by the two methods. There are, however, discrepancies that could be noted, especially in the drop weight method, due to factors which cannot be avoided, but these are so rare that they could be overlooked for practical purposes. In order to avoid repetitions of some general facts relative to surface tension in these and later experiments, discussion will be confined to the results obtained by the film method. The results obtained by the drop weight method are presented in each case for the sake of comparison, and in order that those who are interested may compare these findings with those of other investigators.

The data in table 4 show that during the first observation, the surface tension of all the uninoculated series, treated and untreated with depressants, was practically the same as at the onset and their pH values remained constant. The inoculated series, however, showed a decrease in surface tension of from 1.6 to 4.8 dynes per centimeter. This decline in surface tension was accompanied by an abrupt drop in hydrogen-ion concentration. A correlation of these changes, however, reveals no systematic variation thus indicating that surface tension has no particular effect upon the growth of this organism.

A slight rise of surface tension over that found in the first observation will be noted in all cultures after forty-eight hours

incubation. This increase, however, did not alter the preceding pH value obtained after the twenty-four-hour period. Later observations show that the surface tension increased in all inoculated series until the fifth day period when a general decline of surface tension was noted. These changes in the surface tension of the inoculated series did not materially alter the pH values found at the second day period.

A general view of the results obtained in table 4 shows that there is an increase of surface tension of from 1.8 to 4.6 dynes per centimeter in all the uninoculated series, treated and untreated with soaps, while their pH values remain constant. It will also be noted that in all the inoculated series, a decrease of surface tension of from 1.6 to 4.5 dynes per centimeter was observed. Considering the pH value as an index to the normal activities of the organism, a slight inhibition was noted in all the cultures containing depressants. Inhibition seems to be more marked in the cultures treated with castor oil soap, with olive soap following and cocoanut soap next. Palmitic soap shows a very slight inhibiting action. This inhibition, however, cannot be attributed to the lower surface tension, since the activities of the organism in the medium containing cocoanut oil soap with a surface tension of about 4 dynes lower on the onset than that of the castor oil soap, is nearer to normal than the latter. It is more highly probable that the inhibiting action is due to the chemical nature of the soap.

Surface tension and the growth of streptococci

Two series of flasks of glucose broth arranged as outlined in the previous experiments were carefully sterilized and inoculated, one with a twenty-four-hour culture of *Streptococcus viridans* and the other with a twenty-four-hour culture of *Streptococcus hemolyticus*. Both series were incubated at 37°C. and observations made after one, two, three, five, seven, and ten days. Uninoculated broth was included to serve as a control. The treatment of the flasks, surface tension, and pH values during periods of observation are shown in tables 5 and 6.

It will be seen from the data in table 5 that the surface tension

TABLE 5

Streptococcus viridans and surface tension

	TREATMENT																			
	Castor 0.006%				Olive 0.06%				Cocoanut 0.06%				Palmitic 0.06%				Control			
	Culture		Control		Culture		Control		Culture		Control		Culture		Control		Culture		Control	
	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH
Film method																				
Age of culture:																				
0 day			40.2	7.0			35.8	7.0			34.9	7.0			35.9	7.0			46.7	7.0
1 day	40.3	4.5	41.0	7.0	34.8	4.5	36.4	7.0	33.5	6.2	35.2	7.0	34.8	4.6	36.4	7.0	45.5	4.4	47.2	7.0
2 days	39.6	4.5	40.8	7.0	35.7	4.5	35.8	7.0	32.0	6.2	35.2	7.0	33.9	4.6	35.8	7.0	44.0	4.4	46.8	7.0
3 days	42.9	4.5	43.5	7.0	37.0	4.5	37.0	7.0	29.2	6.2	35.2	7.0	35.9	4.6	36.9	7.0	45.0	4.4	47.0	7.0
5 days	40.7	4.5	41.8	7.0	36.1	4.4	37.4	7.0	31.4	6.0	35.5	7.0	36.2	4.6	36.5	7.0	46.1	4.4	47.2	7.0
7 days	41.0	4.5	41.6	7.0	36.9	4.5	37.2	7.0	31.8	6.0	37.8	7.0	36.4	4.6	36.5	7.0	45.0	4.4	47.2	7.0
10 days	42.4	4.4	42.7	7.0	38.7	4.6	38.5	7.0	31.0	5.8	38.6	7.0	37.3	4.6	37.4	7.0	44.7	4.4	48.2	7.0
Drop weight method																				
Age of culture:																				
0 day			53.2				50.9				47.9				50.9				61.0	
1 day	53.6		54.8		48.3		52.1		45.5		48.7		51.3		52.1		56.8		61.1	
2 days	49.6		53.7		49.3		49.0		47.8		49.6		49.4		49.6		54.7		60.8	
3 days	54.4		54.2		50.8		51.0		48.0		49.6		50.5		50.7		56.9		60.6	
5 days	52.7		54.6		49.6		50.7		48.2		49.8		52.6		50.3		58.2		61.0	
7 days	53.4		53.9		49.7		50.3		47.8		49.5		52.0		49.8		56.8		62.1	
10 days	56.5		56.4		52.0		52.2		46.4		50.7		52.9		52.6		56.2		62.1	

of all uninoculated flasks, treated and untreated with depressants, remained practically the same during the first twenty-four hours while their pH values also remained constant. During the same period, it will be noted that there was a decrease of surface tension of from 0.5 to 4.2 dynes per centimeter in all the series inoculated with *Streptococcus viridans*. This decline in surface tension was accompanied by an increase in hydrogen-ion concentration. The decrease in pH values, however, is not in proportion to the lowering of surface tension or to the surface tension at the onset. In fact, during the first twenty-four hours, the organism has practically reached its maximum development in most of the inoculated series, with varying surface tensions. The cocoanut oil series, showed a marked inhibition but the retarded growth cannot be attributed to surface tension. A discussion of this fact will be found later in this text.

While a marked lowering of surface tension is again noted after forty-eight hours in all the inoculated series, the pH values remained practically the same as in the previous observation. More or less irregular surface tension measurements were obtained after that time but the hydrogen-ion concentrations practically remained the same in all cases as those found after twenty-four hours.

A general examination of the results in table 5 will show that a rise in surface tension of from 1.5 to 3.7 dynes per centimeter was observed in all the uninoculated series, treated and untreated with depressants, after ten days incubation. Their pH values, however, remained constant in all cases. It will also be noted that after twenty-four hours there was an increase of surface tension in most of the inoculated series containing depressants with the exception of the cocoanut oil series which gradually lowered in surface tension until the end of the experiment. The inoculated series containing no depressants showed also a decrease of surface tension of 2.0 dynes per centimeter at the end of the ten-day period. It will be noted that the rise in surface tension of the cultures containing depressants is almost parallel with the rise in surface tension of their respective controls. This would indicate that the products of metabolism of the organisms due

TABLE 6

Streptococcus hemolyticus and surface tension

	TREATMENT																			
	Castor 0.006%				Olive 0.06%				Cocoanut 0.06%				Palmitic 0.06%				Control			
	Culture		Control		Culture		Control		Culture		Control		Culture		Control		Culture		Control	
	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH
Film method																				
Age of culture:																				
0 day			38.9	7.1			35.8	7.1			34.4	7.1			35.0	7.1			44.4	7.1
1 day	38.0	5.0	37.9	7.1	34.5	4.8	34.5	7.1		7.1	34.5	7.1	34.5	4.8	34.8	7.1	40.2	4.8	44.1	7.1
2 days	40.3	4.8	40.0	7.1	35.9	4.8	37.1	7.1	30.2	6.4	34.1	7.1	36.5	4.8	37.1	7.1	43.1	4.6	45.2	7.1
3 days	40.1	4.8	41.2	7.0	36.6	4.8	37.4	7.1	29.7	6.4	34.8	7.1	36.8	4.8	37.9	7.1	43.9	4.6	45.7	7.1
5 days	41.3	4.6	41.0	7.0	37.0	4.8	37.3	7.1	30.3	6.4	36.0	7.1	37.8	4.6	37.6	7.0	44.7	4.6	45.8	7.1
7 days	41.0	4.6	41.1	7.1	37.4	4.6	37.1	7.1	30.5	6.4	36.1	7.0	37.8	4.6	37.4	7.0	44.9	4.4	45.9	7.1
10 days	41.3	4.6	41.7	7.0	38.5	4.6	38.4	7.1	31.1	6.4	36.5	7.1	36.6	4.6	36.8	7.0	43.6	4.4	45.9	7.1
Drop weight method (dynes)																				
Age of culture:																				
0 day			52.0				50.9				44.4				49.1				59.4	
1 day	41.7		50.2		46.9		46.0				43.1		47.6		47.9		50.0		55.8	
2 days	52.2		52.6		48.4		48.4		40.3		43.8		47.2		50.0		51.6		59.0	
3 days	53.3		53.0		48.8		52.0		42.1		45.0		47.4		50.8		52.6		59.8	
5 days	49.7		52.9		49.0		51.9		42.4		44.7		48.0		49.8		53.4		60.1	
7 days	51.9		52.6		49.4		49.8		42.3		44.9		48.0		47.9		53.2		60.7	
10 days	53.0		53.4		49.5		53.2		41.7		42.9		47.3		46.5		52.0		61.2	

to their activities did not alter the surface tension of the medium. In this particular case, the result coincides with the findings of Marshall (1924) who states that no change in surface tension of infusion broth or of synthetic medium results as a consequence of bacterial growth.

The growth of the organism as measured by the pH values was practically normal in the castor, olive, and untreated series, the maximum having been reached during the first twenty-four hours. A very slight inhibition which accompanied the development of the organism from the second to the last day of the experiment was noted in the palmitic acid series, while a very marked inhibition was observed in the cocoanut oil series. In view of the fact that the cocoanut series produced the lowest surface tension, on first thought it may appear that the inhibition of growth in the cocoanut series is due to the reduction of surface tension. Referring to table 3a, 3b and 3c it will be found that a surface tension of 34.9 dynes per centimeter was obtained with palmitic soap which should not inhibit growth of either *Streptococcus viridans* or *Streptococcus hemolyticus*. We can therefore conclude that the inhibition in the cocoanut series is due to the nature of the depressants rather than to the lowered surface tension. In fact there seems to be no correlation between surface tension and the growth of *Streptococcus viridans*.

The data obtained with *Streptococcus hemolyticus* presented in table 6 are in such close harmony with the data for *Streptococcus viridans* presented in table 5 that a separate discussion is not warranted. It may be considered that the behavior of these organisms is so nearly identical in regard to surface tension that a single discussion will serve for either. A careful examination of table 6, will show that there was no growth observed in the cocoanut series during the first twenty-four hours but this is probably due to a lack of mass inoculation.

Surface tension and the growth of staphylococci

Four series of broths containing depressants were arranged as indicated in previous experiments. These series were inoculated, one with a strain of *Staphylococcus aureus* isolated from a case

TABLE 7

Staphylococcus aureus and surface tension

	TREATMENT																			
	Castor 0.006%				Olive 0.06%				Cocoanut 0.06%				Palmitic 0.06%				Control			
	Culture		Control		Culture		Control		Culture		Control		Culture		Control		Culture		Control	
	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH	S. T. Dynes	pH
Age of culture:																				
0 day			45.9				43.9	7.2			38.8	7.2			42.2	7.2			51.4	7.2
1 day	43.8	6.4	45.8	7.2	39.1	6.5	lost	7.2	34.6	6.8	38.7	7.1	38.9	6.5	42.2	7.1	50.4	5.4	51.3	7.2
2 days	42.0	5.8	46.0	7.2	39.1	5.8	42.5	7.2	32.4	6.8	39.0	7.2	39.5	6.0	42.6	7.1	49.3	5.4	51.5	7.2
3 days	41.0	5.6	45.9	7.2	40.5	5.8	42.7	7.2	31.0	6.3	38.7	7.2	40.2	6.1	42.7	7.2	49.1	5.4	51.4	7.2
5 days	43.7	5.8	45.9	7.2	40.4	6.0	43.5	7.2	30.5	5.6	39.1	7.2	40.6	6.3	43.1	7.2	50.1	5.6	51.4	7.2
7 days	40.7	5.8	46.0	7.2	43.4	6.0	43.3	7.2	29.5	5.6	39.8	7.2	41.3	6.0	44.0	7.2	48.1	5.6	51.5	7.2
10 days	41.2	5.8	46.9	7.2	39.9	5.8	44.9	7.2	29.7	5.6	40.7	7.2	40.7	6.0	45.4	7.2	46.8	5.4	52.8	7.2

of navel ill, a second with a strain of *Staphylococcus aureus* recently isolated from a case of furunculosis, the third and fourth with strains of *Staphylococcus albus* isolated from cases of acne infection. All four series, were incubated at 37°C. and observations made after one, two, three, five, seven and ten days. In these series only the film method of measuring the surface tension was employed.

The results obtained from these four series are so nearly parallel that a single table will serve for all, and a separate discussion of each one is not warranted. Table 7 shows the arrangement of the flasks, pH, and surface tension over the course of the experiment.

It is seen from the data in table 7 that while the surface tension of the uninoculated flasks, treated and untreated with depressants, remained practically the same during the first twenty-four hours, the inoculated series showed a marked decrease in all cases. It will be noted that the cultures containing depressants decreased by from 2.1 to 4.8 dynes per centimeter while the inoculated control decreased by 1 dyne. This change in surface tension was not in proportion to the extent of development of the organism as noted from the pH values.

A comparison of the hydrogen-ion concentration of all the cultures shows that the maximum growth of the organism is already reached in the untreated series during the first twenty-four hours, while in all cultures containing depressants inhibition is noted. This inhibition during the first twenty-four hours is most marked in the culture containing cocoanut oil soap. This, however, could not be attributed to surface tension because later observations show that the growth of the organism in this series is nearer to normal than in the case of the cultures containing depressants and a higher surface tension at the onset.

During the second observation lowering of surface tension was noted again in all cultures but the decrease was comparatively less than that obtained during the first twenty-four hours. A marked change was also observed in the growth of the organism. Later observations show that the change in surface tension was more or less irregular in the cultures containing depressants.

This irregularity, however, did not affect the growth of the organism. In most cases, the maximum development of the organism in the broths containing depressants was reached after the third day and practically remained the same until the end of the experiment so that the limit of growth was attained before any irregularities in surface tension were noticed.

A general survey of the results in table 7 will show that the surface tension in all cultures decreased by from 2.1 to 9.1 dynes per centimeter. It will also be noted that all uninoculated flasks, treated and untreated with soaps, increased in surface tension by from 1 to 3.2 dynes while their pH values remained constant.

An interesting result was brought out by this experiment. Table 7 shows that while inhibition is seen in all cultures containing depressants, palmitic oil soap showed the most marked effect. This is very different from the results obtained in the experiments with *Diplococcus pneumoniae* and streptococci. Castor oil soap showed the most marked inhibition on the growth of *Diplococcus pneumoniae* while cocoanut oil soap inhibited the growth of streptococci more than any of the other depressants employed. The inhibition of growth of *Staphylococcus aureus* in the case of palmitic soap is not due to surface tension. This point is established by the fact that the best growth observed, aside from the control, was in the cocoanut oil soap which gave the greatest reduction in surface tension.

The data in the experiments with the staphylococci indicate that there is no correlation of surface tension with development of the organisms. There is a strong indication of inhibition of growth due to the chemical nature of the depressants.

SUMMARY AND CONCLUSIONS

Seven organisms were studied in order to observe their behavior when grown in media of varying surface tension. These organisms consisted of *Diplococcus pneumoniae*, *Streptococcus viridans*, *Streptococcus hemolyticus*, and four strains of staphylococci.

The surface tension was determined by the method of Fahrenwald and the drop weight method. The two methods do not give the same value, for the surface tension of a liquid, the drop

weight method yielding, in general, results about 12 dynes per centimeter higher. It is not possible at present to say which is the more reliable, but the results obtained by the method of Fahrenwald are less subject to variation and error in manipulation.

Considerable variation in growth was observed in the broths containing the depressants. In general there was a marked inhibition of growth during the first twenty-four hours. This inhibition was in a good many instances largely overcome within three days but in few cases did the growth equal that in the control tubes.

The inhibition was not directly associated with surface tension. In many instances, the greatest inhibition occurred at higher surface tensions while the broths of the lowest surface tension gave the maximum development in the reduced series. This indicates that inhibition was due to the chemical nature of the depressant rather than to reduced surface tension.

There is nothing to indicate, within the limits of these experiments, that surface tension is a factor in the development of the organisms studied.

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BACTERIA PREVALENT IN SWEETCORN CANNING

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In an investigation of the decomposition of sweetcorn (James, 1925), it was found that the initial bacterial count upon the ears freshly cut in the field was about 30,000 organisms per kernel when grown on plates incubated at 30°C., and an average of one organism per kernel when the incubation temperature was 55°C. Dilution counts made upon samples removed from the corn as it passed through the processes of a commercial cannery showed an increase from 135,000 organisms grown at 30°C. and two at 55°C. on corn just after being husked, to 22,500,000 organisms at 30° and two at 55° on corn just before it is preheated to 185°F. This "preheat" reduced the 30°C. count to 40, but did not affect the 55°C. count. Fresh, unhusked corn, packed in a barrel which had been placed in the center of a small pile of corn husks, underwent marked heating and rapid spoilage. Observations extending over four days showed: First, a rise in temperature to 47°C. or above after two days, ending on the fourth day at 51°C.; second, a marked increase in organisms growing at 30°C., followed by a decrease to a value below that of fresh corn; and third, an increase, especially after 47°C. was reached, of organisms growing at 55°C.

Piles of corn placed in the cannery bin at night or on Sunday for use the following day, were hot and steaming by morning, with temperatures as high as 50°C. and about 580,000 viable organisms per kernel. Similar determinations on corn which had remained in the farmer's wagon over night in a light rain, showed a temperature of 55°C., with a 30° bacterial count of 10,000,000 organisms per kernel, and a 55° count of 50 organisms per kernel.

During this investigation, many organisms were isolated for detailed study. In the isolation of these organisms, it was desired, first, to study the types which were present in large or predominating numbers, and which would probably contribute more to the spoilage of the sweetcorn than those present in smaller numbers, and second, to obtain and study any particular organisms which might be significant as a cause of spoilage of the canned product. Donk (1919) reports the isolation of several organisms from canned products, and Prescott and Underwood (1898), in an extensive paper on the souring of sweetcorn, report the isolation of organisms from cans of sour corn and also from corn before being canned, concluding that "Bacteria which produce sour corn are found on the kernels and beneath the husks of the corn as it comes from the field." Wahl (1906) and Cheyney (1919) report upon the isolation and identification of organisms found in canned foods.

EXPERIMENTAL

The organisms included in this report were obtained from freshly gathered corn, corn as it passed through the cannery, corn packed in a barrel and allowed to heat, corn piled in the cannery bin over night, and corn remaining in the farmer's wagon over night.

The total number of organisms per cubic centimeter was so high that the necessary dilutions allowed only those present in large numbers to appear with marked frequency upon the plates. Representative colonies were fished from plates incubated at 30°C. and 55°C.

A study of their important morphological and cultural characteristics divided the 66 cultures selected into 18 groups. The organisms within each group are of the same general type, therefore one typical organism has been selected to represent each entire group, and reference hereafter will be made to the representative organism as indicated by number. In table 1 are listed the numbers of the groups, the number of original cultures included in each group, the sources and optimum temperature of the cultures, and the general type of microörganism.

TABLE 1

Sources of original cultures

KEY NUMBER OF ORGANISM REPRESENTING GROUP	NUMBER OF ORIGINAL CULTURES INCLUDED IN GROUP	SOURCE OF CULTURES	OPTIMUM TEMPERATURE	IDENTIFICATION
			°C.	
CI_3	1	Fresh corn	30	Flavobacterium
CI_8	5	Fresh corn; corn from screens; corn in barrel 4 days; corn loaded on wagon over night	50	*Bac. mesentericus* (Flügge)
CI_{14}	2	Fresh corn	30	*Bac. subtilis* (Ehrenberg)
CI_{15}	3	Fresh corn, predominating at base of ear	30	Achromobacter
CI_{16}	2	Fresh corn, predominating at middle of ear	30	*Bac. subtilis* (Ehrenberg)
CI_{17}	3	Fresh corn, predominating at tip of ear	37	Achromobacter
CII_3	1	Corn packed in barrel, 2 days	30	Flavobacterium
CTI_1	1	Fresh corn	50	
CTI_7	2	Fresh corn; corn in barrel 3 days.	50	*Bac. Agri.* (Ford)
CTI_{13}	2	Fresh corn; corn after cut from cob	37	
CTI_{16}	1	Fresh corn	50	*Bac. cohaerens* (Gottheil)
CTI_{19}	1	Fresh corn	30	*Aerobacter aerogenes* (Escherich)
$CTII_{16}$	33	Fresh corn—7 cultures; preheated brine—2 cultures; corn from screens—1 culture. Corn preheated, just before sealing can—4 cultures Corn in barrel, 1 day—2 cultures; corn in barrel, 2 days—1 culture; corn in barrel, 3 days—9 cultures; corn in barrel, 4 days—4 cultures; corn loaded on wagon over night—2 cultures	50	*Bac. subtilis* (Ehrenberg)
$CTIII_4$	2	Corn in barrel 1 day—1 culture; corn in barrel 2 days—1 culture	50	

TABLE 1—*Concluded*

KEY NUMBER OF ORGANISM REPRESENTING GROUP	NUMBER OF ORIGINAL CULTURES INCLUDED IN GROUP	SOURCE OF CULTURES	OPTIMUM TEMPERATURE	IDENTIFICATION
			°C.	
$CTIII_{17}$	1	Corn in barrel 3 days	50	
$CTII_{41}$	2	Corn, preheated, just before sealing can; corn in barrel, 4 days	37	*Bac. cereus* (Frankland)
$CTIV_{10}$	2	Corn in bin over night	50	
$CTIV_{13}$	2	Corn in barrel 4 days; corn in bin over night	50	*Bac. subtilis* (Ehrenberg)

The classification arranged by a committee of the Society of American Bacteriologists (Bergy, 1923) has been used as a basis for these comparisons. In testing for spore formation, it was found that 57 strains, incubated on agar slants at their optimum temperature for twenty-four hours, survived when heated to 80°C. for twenty minutes.

Gram stains were made by the use of Sterling's Gentian Violet, Lugols' Iodine solution, alcohol and dilute basic Fuchsin. Several of the spore-forming cultures appeared Gram negative when stained after twenty-four hours incubation at their optimum temperature. In checking these after six hours incubation, two cultures, CTI_1 and $CTIII_{17}$, still proved to be Gram negative. Carbohydrate fermentation and gelatin liquefaction tests were incubated five days, the latter then placed in the icebox for final readings.

Of the 12 organisms isolated from fresh corn, five showed an optimum growth temperature of 30°C., five an optimum of 50°, and two an optimum of 37°. It is interesting to note that of the five growing best at 30°, one, CTI_{19}, an *Aerobacter aerogenes* strain, was isolated from a plate incubated at 55°C., and of the five with an optimum of 50°C., one, CI_8, a mesentericus type, was isolated at 30°C. This latter organism was isolated on several occasions. The group containing the largest number of original strains, $CTII_{16}$, was made up of the subtilis type of organisms and was found at almost every stage or process that

the corn passes through. Of the five original cultures obtained from the preheated corn just before the cans were sealed and processed, which is the point at which any organism endangering the success of the cook would be found, four fall within this group. In this connection, it is recalled that the preheat of the corn killed over 99 per cent of the organisms growing at 30°C., but did not affect the numbers which grew at 55°C. Two out of 22 cultures isolated from the corn in the barrel were obtained during the first two days of storage, while the temperature was below 47°C., and the remaining 20 were obtained after the temperature had remained at or above 47°C. for four days. These all fell within this same group of subtilis-like organisms.

Since 13 of the 18 types of organisms isolated formed spores abundantly during forty-eight hours incubation, and since certain types of these have been shown to be present on fresh corn, to multiply during storage of the corn and to be unaffected by the preheat to 185°F. the question naturally arises as to whether or not these or any other forms present in the corn would survive the cooking process of about seventy-five minutes at 250°F. Since strains of the same organism might vary in resistance, thermal death point determinations were made on all 57 spore-forming cultures.

In the determination of the thermal death points, the method reported by Bigelow and Esty (1920) and later modified by Esty and Williams (1924), namely, that of immersing sealed Pyrex tubes containing the bacterial suspension in a constant temperature oil bath was used with two modifications: First, 10 tubes were removed from the oil bath for each determination, instead of the 30 recommended, and second, the temperature was maintained in the bath within 1.0°C. of the desired point. These changes were made because the temperature-time figures obtained were to be compared with those of the ordinary commercial process, wherein such accuracy as that used by Bigelow and Esty is not observed. Suspensions containing 1,000,000 spores per cubic centimeter were made in glucose broth, pH 7.0, with bromcresol purple added as indicator.

Tests in the oil bath were made at 250°, 245° and 240°F., and

TABLE 2

Resistance of spores at 250°F.

CULTURE NUMBER	GROUP	PREHEAT	TIME OF EXPOSURE AT BATH TEMPERATURE			
		15 seconds	1 minute	2 minutes	3 minutes	4 minutes
CTI_2	$CTII_{16}$	0*	100*	100	100	100
CTI_{18}	$CTII_{16}$	0	100	100	100	100
$CTII_{12}$	$CTIII_{41}$	0	100	100	100	100
$CTIII_{40}$	$CTII_{16}$	0	70	90	100	100
$CTIV_7$	$CTII_{16}$	100	100	100	100	100
$CTIV_{13}$	$CTIV_{13}$	0	100	100	100	100

* The figures indicate per cent killed.

TABLE 3

Resistance of spores at 245°F.

CULTURE NUMBER	PREHEAT	TIME OF EXPOSURE AT BATH TEMPERATURE			
	15 seconds	1 minute	2 minutes	3 minutes	4 minutes
CTI_2	0	100	100	100	100
CTI_{18}	0	100	100	100	100
$CTII_{12}$	0	100	100	100	100
$CTIII_{40}$	0	10	100	100	100
$CTIV_7$	0	100	100	100	100
$CTIV_{13}$	0	0	100	100	100

TABLE 4

Resistance of spores at 240°F.

CULTURE NUMBER	PREHEAT	TIME OF EXPOSURE AT BATH TEMPERATURE			
	15 seconds	1 minute	2 minutes	3 minutes	4 minutes
CTI_2	0	100	100	100	100
CTI_{18}	0	100	100	100	100
$CTII_{12}$	0	100	100	100	100
$CTIII_{40}$	0	0	20	100	100
$CTIV_7$	0	80	100	100	100
$CTIV_{13}$	0	0	100	100	100

only those organisms which survived the 15 seconds preheat at 240°F. in all 10 tubes are included in this report. Results are listed in tables 2, 3 and 4.

Organism $CTIII_{40}$, which falls within the largest group of subtilis-like organisms, $(CTII_{16})$, was the most resistant of all the strains. $CTIV_{13}$, also a subtilis-like organism, but fermenting lactose, was able to survive 245°F. for one minute, and 250°F. only through the fifteen seconds preheat. It is interesting to note that four of the cultures included in tables 2, 3 and 4 are subtilis-like organisms with an optimum temperature of 50°C., yet not all possess the same powers of resistance to heat.

The maximum temperature attained in the center of a can of corn and the length of time the corn is subjected to any one temperature were readily estimated from the curves of heat penetration published by Bigelow (1920), p. 120. Thus, it was found that in retorting at 250°F., the center of the can attained a maximum temperature of about 246°F., that a temperature of 245°F. or above was maintained for about twelve minutes, and a temperature of 240°F. or above for about thirty-four minutes. Similar time coefficients have been estimated for other retort temperatures as follows:

TEMPERATURE OF RETORT	MAXIMUM TEMPERATURE ATTAINED IN CAN	TIME AT 245°F.	TIME AT 240°F.	TIME AT 235°F.
°F.		*minutes*	*minutes*	*minutes*
260	250	16	25	32
255	248	18	28	35
250	246	12	34	44
245	243		39	59
240	240			78

Referring now to the organisms listed in tables 2, 3 and 4, the most resistant, $CTIII_{40}$, was not killed by a short exposure to 250°F., but at 245° it was killed within two minutes. Thus, it would not survive the commercial cooks at 260°, 255°, and 250°F. This organism likewise did not survive the test at 240°F. over three minutes and, therefore, would not survive in a can of corn which is processed at 245°F. Since this organism was killed at 240°F. within three minutes, it is safe to assume that it would not survive an exposure in corn in a commercial retort at 235°F. for seventy-eight minutes. In a similar manner, culture $CTIV_{13}$,

in which all spores were killed at 240°F. within two minutes, would not survive retorting in a can of corn at 260°, 255°, 250°, or 245°F., and probably not at 240°F.

While similar comparisons of the other organisms show that not one of the cultures isolated would survive any of the processes ordinarily given a can of corn in a commercial retort, it must be noted that the thermal death points of the most resistant organisms are considerably below those of strains isolated from canned products reported by other workers. In experiments previously mentioned, fresh corn piled in the husking sheds was shown to heat rapidly, with a resultant marked multiplication of microörganisms which, though thermophilic, were not highly heat resistant. The experiments were not continued long enough to permit of the selective action which would be encountered in long continued use of heated corn. Since in the factory where these studies were conducted the corn was handled with care and dispatch, allowing little opportunity for the unhusked corn to accumulate in piles and heat, it is not surprising that no highly resistant spore-formers were isolated from the corn in the course of canning. Inspection of many other corn canning factories showed that the conditions in the factory where these studies were conducted were representative of the average throughout the Middle West.

SUMMARY

Sixty-six strains have been isolated as representing the types of organisms frequently present upon sweetcorn during the processing and handling, and during spoilage. These were isolated from freshly cut ears of corn, from samples of the corn as it passed through the cannery, and from stored corn.

On the basis of cultural study, these organisms were divided into 18 types. Four were organisms allied to *Bac. subtilis* (Ehrenberg); of the remaining strains one resembled *Bac. mesentericus* (Flügge); another *Bac. cereus* (Frankland); a third *Bac. Agri* (Ford), a fourth *Bac. cohaerens* (Gottheil), and a fifth *Aerobacter aerogenes* (Escherich); two others were similar to the Achromobacter; two to Flavobacterium; and five were not readily identifiable. Eleven produced spores.

The largest group, made up of 33 original strains, contained a *Bac. subtilis* organism which was present on fresh corn at every step in the process of canning and was unaffected by the preheat to 185°F.; it was present in large numbers in "heating" corn; and certain strains of this type were moderately heat-resistant.

Thermal death point determinations of 57 strains which produced spores revealed only six which would withstand in the 10 tubes tested a temperature of 240°F. maintained for fifteen seconds. Comparison of the thermal death points determined at 250°, 245° and 240°F., with the curves of heat penetration into corn when processed at those temperatures, made it apparent that none of the organisms obtained would withstand the cooks recommended to the commercial canner. Thus, corn handled with care and dispatch and, when canned, given the full process recommended, would not contain highly resistant thermophiles.

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CAN B. COLI BE USED AS AN INDEX OF THE PROPER PASTEURIZATION OF MILK?

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Testing for the colon bacillus in connection with the efficiency of pasteurization is a procedure which is at present somewhat in disrepute. This is due to the fact that the observations of several investigators (De Jong and De Graff; Shippen, 1915; and Ayer and Johnson, 1915), who found that certain strains of this group of organisms will withstand the pasteurization temperature (145°F. for thirty minutes),[1] have been confirmed by experience, and there is no longer any doubt that a few cells of certain strains of colon organisms will survive pasteurization.

It is not the purpose of this paper to question this fact but rather to point out how, in spite of it, a test for colon group organisms in pasteurized milk may be used to check up on the performance of pasteurizing plants. In this discussion it will be considered that a properly pasteurized milk is one that has been heated sufficiently to destroy all pathogenic organisms (at least 143°F. for thirty minutes); promptly cooled to a temperature which practically stops growth of bacteria (40°F.); and introduced into properly processed containers without recontamination. The term *B. coli* will be used in its broader sense as including all members of the group of non-spore-forming aerobic bacilli which ferment lactose with the formation of gas.

The tests for *B. coli* which constitute the data presented here were made in accordance with the procedure outlined in a subsequent paper.[2] Tests were made on decimal dilutions and the

[1] A temperature which can not be exceeded without materially injuring the product both from a dietetic as well as a commercial point of view.

[2] Gentian violet lactose.

results recorded as 0, 1, 10, 100, etc., which indicate roughly the probable number of *B. coli* per cubic centimeter of milk. The result of a single determination is not considered as having any particular significance, except as will be brought out further on; the number of *B. coli* per cubic centimeter should however not exceed a certain limit in a properly pasteurized milk. The use of the *B. coli* test in connection with the efficiency of pasteurization which I wish to suggest must be based on a series of tests made uniformly over a period of not less than six months.

In the course of a bacteriological study of the milk supply of the city,[3] it was noticed that there was considerable difference in the pasteurized milk from different plants with respect to the *B. coli* content. This difference was so striking that I decided to make a detailed study of our data. There were available for the year 1924 *B. coli* determinations of the pasteurized milk of all the plants in the city covering the period April 27 to December 20. The data on some of the plants were not sufficient to include them in this study but on sixteen plants we had twenty-five or more determinations well distributed over the period, and these were selected for study.

The method of study was to determine the percentage of the total number of samples which showed 0, 1, 10, 100, etc., *B. coli* per cubic centimeters. These percentage figures were plotted on cross-section paper, as shown in the accompanying charts, and in order to get some figure which would show the relative standing of the plants with respect to the *B. coli* content of the pasteurized milk, these percentage figures in each case were multiplied by the number indicating the probable number of *B. coli* per cubic centimeter (0, 1, 10, 100, etc.) which the percentage represented. These figures were then added and the resulting sum recorded as the *B. coli* index. Obviously this figure can not be used, as such, to measure efficiency of pasteurization, but if it is admitted that viable *B. coli* should be practically eliminated from a properly pasteurized milk the figure can be used to arrange

[3] A comprehensive bacteriological study of the milk supply of Baltimore. Jos. C. Swenarton and J. H. Shrader. Abstract of Preliminary Paper, American Journal of Hygiene, May, 1925.

TABLE 1

Dairies listed in order of efficiency of pasteurization as determined by the B. coli index; notes on heating and cooling charts, and bacterial condition of processed bottles

1924		1925				
Dairy and colon index		Dairy and colon index	Notes on heating and cooling charts	Approximate bacteria per bottle: Average	Maximum	Minimum
A 87		A 10	Heating temperature regular 143° to 145°F. Cooling temperature regular below 40°F.	247	630	15
B 141		B 153	Heating temperature regular 142° to 144°F. Holding period regular. Cooling very regular at 40°F.	2,850	43,000 31,000	0
C 467		C 150	Heating very regular 144° to 145°F. Cooling somewhat irregular but mostly below 40°F.	73	320	0
D 504	90 per cent supply	D 194	Heating regular 143° to 145°F. Cooling regular between 30° to 40°F.	3,676	30,000	0
E 827		E 625	Heating regular 144°F. Cooling irregular around 40°F.	6,010	120,000	0
F 884		F 840	Heating mostly very regular but there are a few exceptions where there is considerable variation at beginning of curve. Heating temperature 143° to 144°F. Cooling somewhat irregular but mostly below 40°F.	73	320	0
G 1,423		I 1,104	Heating irregular 140° to 150°F. Time of holding variable. Cooling fairly regular above 45°F.	407	4,600	0
H 1,683		M 1,568	Heating irregular 140° to 150°F. Cooling fairly regular below 40°F.	8,593	55,000	0

TABLE 1—*Concluded*

1924 Dairy and colon index		1925 Dairy and colon index	Notes on heating and cooling charts	Approximate bacteria per bottle: Average	Maximum	Minimum
I 5,863		K 1,718	Heating fairly regular 143°F to to 145°F. Time of holding irregular. Cooling fairly regular between 40° to 50°F.	2,043	15,000	35
J 8,839		O 4,679	Heating irregular 140° to 145°F. Some charts have appearance of being fudged. Cooling irregular 40° to 50°F.	502	6,700	0
K 8,979		H 6,690	Heating fairly regular 143° to 148°F. Time of holding regular. Cooling regular 40°F.	10,423	98,000	0
L 10,317		J 7,874	Heating irregular 140° to 142°F. Holding time irregular. Cooling regular 40°F.	5,011	110,000	5
M 16,422		P 14,030	Heating irregular 135° to 142°F. Time of holding irregular. Cooling very irregular	285	1,700	0
N 25,687		L 15,670	Charts look as though they were made up for the occasion	14,010	85,000	0
O 46,691		N 33,963	Charts undoubtedly fudged	19,053	130,000	0
P 50,094		G 76,397	Heating irregular 140° to 142°F. Time holding irregular. Cooling irregular 40° to 42°F.	57,268	900,000	0

the plants in their order of efficiency. This was done, and the first column of the table shows this arrangement for the 1924 period, together with the index figures. This arrangement fitted in so well with our preconceived idea of the performance of the plants that we decided to repeat the work this year.

During the present year determinations were made in a manner similar to that of 1924 except that the samples were taken from the bottled milk after being held eighteen to twenty-four hours at 45°F., whereas in 1924 the samples were taken in sterile bottles from the bottle filling machine, refrigerated and examined the same day. The period studied this year was from March 15 to October 24. The resulting arrangement of the same plants for the 1925 period is shown in the second column of the table. In addition there are included in the table notes made after a study of the heating and cooling charts from these dairies for the month of May and June. The table includes a column showing the approximate number of bacteria found in the processed bottles from different plants as determined by averaging the results on 15 or more bottles,[4] and also the maximum and minimum results on individual bottles.

DISCUSSION

An examination of the table shows that the plants studied fall into two general groups: (1) Those manifesting regularity of procedure (above the heavy line), and (2) those which are irregular in performance (below the heavy line). This division is justified both from the standpoint of the colon index and the pasteurization charts. Also the relative position assigned to all the plants by the colon index seems to be entirely justified, with the possible exception of the plant G which according to these charts would be expected to occupy a position near the middle of the series. However, it is entirely possible that the reason for this exception is improperly processed bottles. While our data on bottles do not seem to indicate any particular relation with the colon index, the bottles from this particular dairy are on the average so dirty as compared to the other dairies that we feel justified in assigning the poor performance of this dairy with respect to the colon index to improperly processed bottles. We

[4] The number of bacteria per bottle is roughly determined by rinsing the bottles with 10 cc. of sterile water and plating the water according to the standard method for estimating the number of bacteria in milk. The results are recorded as the approximate number of bacteria per bottle.

have no data with respect to *B. coli* in bottles, but we are inclined to believe that while in many instances even in the case of the better dairies the bottles are far from sterile, the organisms present are for the most part spore bearers and therefore have no effect on the colon index. On the other hand, there are undoubtedly cases where the bottle processing is faulty and a poor colon index results from this cause. However, this does not minimize the value of the index for use on bottled milk, for, as was stated at the beginning of this paper, the proper processing of bottles is an essential of a properly pasteurized milk.

The data presented in this table indicate quite clearly that the colon content of pasteurized milk which has been kept well refrigerated until examined may be of decided value in checking up on the performance of pasteurizing plants. Of further interest in this connection are the curves or frequency polygons shown in figures 1 and 2. In the first chart it will be noted that the six dairies (A, B, C, D, E and F) as would be expected, all have very similar curves, with a very decided mode on the zero (0) abscissa. This is the curve of a properly pasteurized milk. A smooth curve would indicate uniform procedure, and the steeper the curve the more satisfactory the pasteurization. Our data are not sufficient to make any further generalizations with respect to these curves, but we are inclined to think that they may have considerable significance with respect to temperature of pasteurization, regularity of procedure, leaky valves, etc. For instance, a curve with two modes might indicate variable procedure; a curve with the mode to the right of the zero (0) abscissa might indicate that the heating was not sufficient or that the valves were leaky. In the 1925 period there was only one plant (G) showing a curve with distinct modes. This plant evidently is irregular both in the pasteurization process proper and in the processing of bottles. There were seven plants with a curve showing the mode removed to the right, The heating and cooling charts from all but one of these dairies show irregularity in procedure. Two plants (J and O) show that the heating temperature is at times as low as 140°F. One plant (P) shows that heating may be as low as 135°F., and two plants (L and N) show fudged

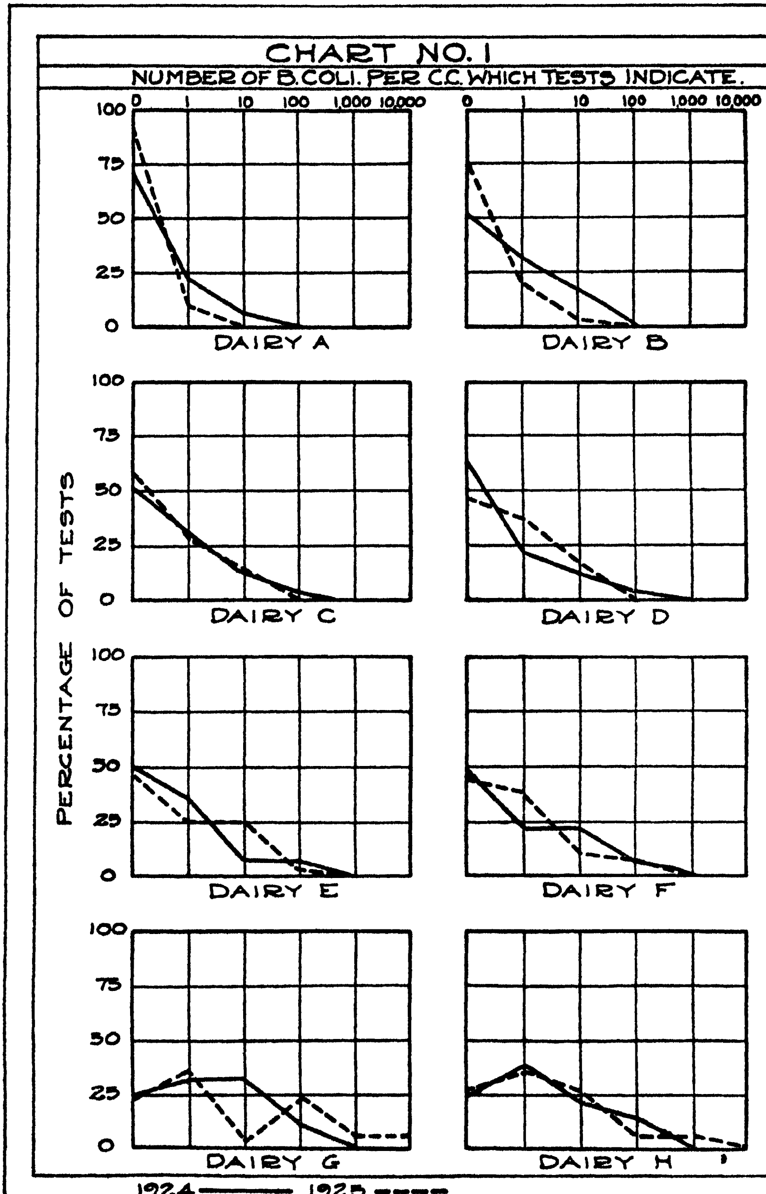
CHART NO. 1
NUMBER OF B. COLI. PER C.C. WHICH TESTS INDICATE.
0
1
10
100
1,000
10,000
100
75
50
25
0
PERCENTAGE OF TESTS
DAIRY A
DAIRY B
DAIRY C
DAIRY D
DAIRY E
DAIRY F
DAIRY G
DAIRY H
1924 ———— 1925 - - - -

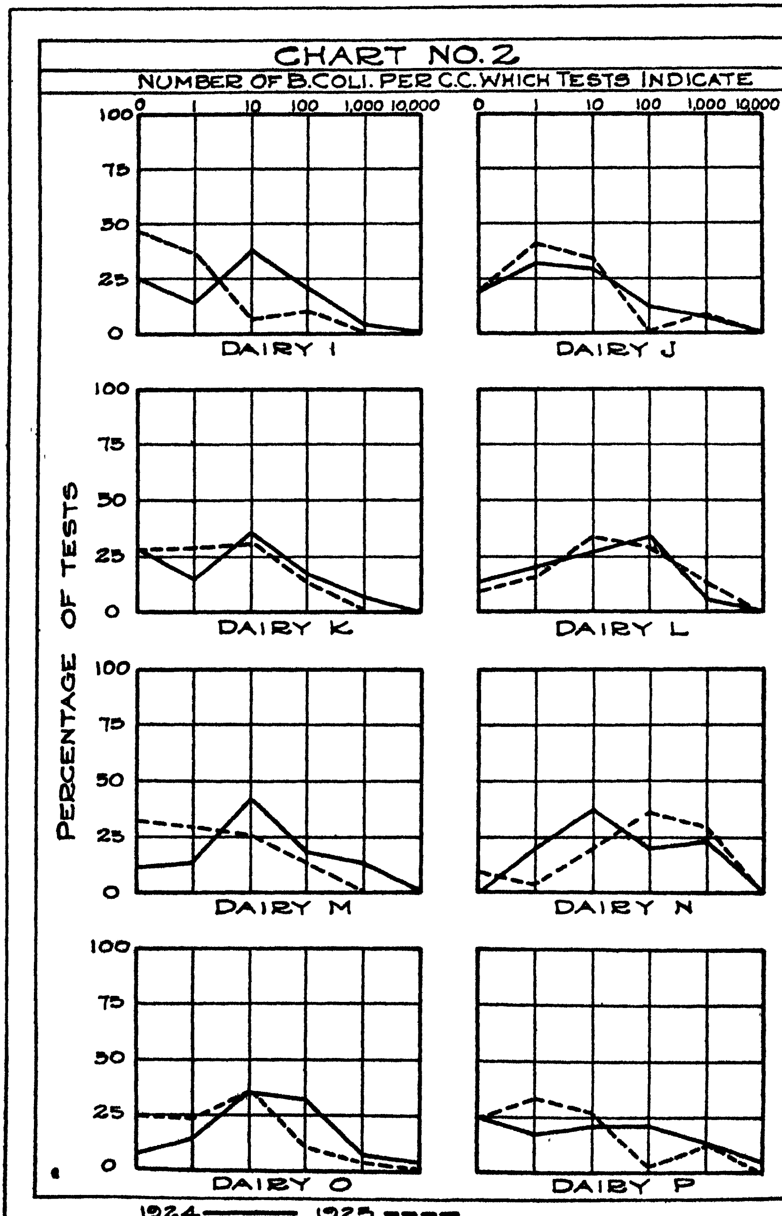
CHART NO. 2
NUMBER OF B. COLI. PER C.C. WHICH TESTS INDICATE
0
1
10
100
1,000
10,000
100
75
50
25
0
PERCENTAGE OF TESTS
DAIRY I
DAIRY J
DAIRY K
DAIRY L
DAIRY M
DAIRY N
DAIRY O
DAIRY P
1924
1925

charts. The pasteurizing charts from the one plant (H) which gave a curve with the mode on the one (1) per cubic centimeter abscissa, shows fairly consistent procedure. This may be a case of leaky valves or improperly processed bottles.

In addition to showing how the *B. coli* test can be used in checking up on the performance of pasteurizing plants, it would be well to set a standard with respect to the number of *B. coli* which might be allowed in pasteurized milk. In this connection it would seem reasonable to assume that the milk pasteurized by the plants A, B, C, D, E and F, shown in table 1, above the rule was for the most part properly pasteurized. These plants handle about 90 per cent of the city supply, and a standard which our figures indicate could be met by these plants should be a reasonable one. The percentage results of our colon tests on these plants are as follows:

PLANT	YEAR	NUMBER OF SAMPLES	PER CENT NEGATIVE IN 1 CC.	PERCENTAGE FIGURES INDICATING THE TOTAL NUMBER OF SAMPLES POSITIVE IN THE QUANTITY OF MILK INDICATED				
				1 cc.	1/10 cc.	1/100 cc.	1/1000 cc.	1/10000 cc.
A	1924	31	71	29 0	6 4	0	0	0
	1925	30	90	10.0	0	0	0	0
	Both	61	80.3	16.4	3.3	0	0	0
B	1924	29	51 7	48.3	17.2	0	0	0
	1925	30	76.7	23 3	3.3	0	0	0
	Both	59	64 4	35.6	10 2	0	0	0
C	1924	32	53.1	46.9	15 6	3.1	0	0
	1925	31	58.1	41.9	12.8	0	0	0
	Both	63	55.5	44 4	14 3	1.6	0	0
D	1924	27	63.0	37.0	14.8	3.7	0	0
	1925	32	46.9	53.1	15.6	0	0	0
	Both	59	54.3	45 7	14.2	3.4	0	0
E	1924	28	50.0	50.0	14.3	7.2	0	0
	1925	28	46.5	53.5	28 5	3.5	0	0
	Both	56	48.2	51.8	21.4	5.3	0	0
F	1924	27	48.2	51.8	29.6	7.4	0	0
	1925	29	44.8	55.2	17.2	7.0	0	0
	Both	56	46.4	53.6	23.2	7.1	0	0

While the problem is not exactly the same, the figures indicate that a standard similar in outline to that recommended for water[5] is applicable.

Following this outline a standard might be worded as follows:

DEFINITION

The standard portion of milk shall be 0.1 cc. The standard sample shall consist of five standard portions of 0.1 cc. each.

STANDARD

1. Of all the standard 0.1 cc. portions examined, not more than 20 per cent shall show the presence of organisms of the *B. coli* group.

2. Occasionally three or more of the five equal 0.1 cc. portions constituting a single standard sample may show the presence of *B. coli*. This shall not be allowable if it occurs in more than (a) 10 per cent of the standard samples when ten or more samples have been examined; or in (b) one standard sample when less than ten samples have been examined.

Referring to the figures for the six plants which we have considered as giving good indication of proper pasteurization, it will be seen that in the 1924 period all but one (F) would have met the first part of the standard. Again in the 1925 period all but one (E) would have met this part of the standard. Using the figures for both the 1924 and 1925 periods, four of the plants (A, B, C and D) meet part one of the standard. These four plants handle about 86 per cent of the supply, while the two plants which do not meet it in one of the two periods each contribute about 2 per cent of the supply. Summarizing these, we have in each of the two periods studied 88 per cent of the supply meeting the first part of the proposed standard. Regarding the second part of the standard we have no definite information since our tests were not made in sets of 5. However, it may be assumed

[5] Report of Advisory Committee on Official Water Standards. Pub. Health Reports, Vol. 40, p. 693, 1925.

that a plant meeting the first part of the standard would meet the second part, since this simply allows within certain limits for an uneven distribution of the positive tests.

SUMMARY AND CONCLUSIONS

1. Sixteen pasteurizing plants were studied with respect to the *B. coli* contents of the pasteurized milk.
2. The *B. coli* content of the pasteurized milk from the different plants was found to vary considerably.
3. The control charts from the plants whose milk was high in *B. coli* show improper heating or irregularity of procedure. There is a definite correlation between *B. coli* content and procedure as indicated by the control charts.
4. A test for *B. coli* in pasteurized milk can be used to good advantage in checking up on plant performance.
5. A standard is proposed for the maximum *B. coli* content of a properly pasteurized milk.

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INDEX TO VOLUME XIII

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